The Use of Irreversible Antagonists as Anti-spasmodic Agents in the Radial Artery

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Medicine by **Michael John Shackcloth**

September 2009

Declaration

This Thesis is the result of my own work. The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or other qualification.

The research/clinical work was carried out in The Cardiothoracic Centre Liverpool NHS Trust, Research Laboratory.

Acknowledgements

The work presented in this thesis was funded jointly by the British Heart Foundation and The Mersey Beat Appeal.

I would like to thank Consultant Cardiac Surgeons Mr Brian Fabri, Mr Walid Dihmis, Mr Neeraj Mediratta, Miss Elaine Griffiths, Mr Abbas Rashid, Mr Aung Oo and Mr Mark Pullan whose patients provided the samples used in this thesis. I would also like to thank the theatre staff for their help in collection of the samples. The advice and help of Ian Whittle, Audit Department Liverpool Heart and Chest Hospital, with the statistical analysis in chapters 2, 3 and 4 is very much appreciated.

I am grateful for the help of Dr Alec Simpson from the Department of Human Anatomy and Cell Biology at The University of Liverpool for his advice and constructive criticism in the preparation of this thesis.

Finally, I am indebted to Dr Alan Connant, formerly of the Department of Human Anatomy and Cell Biology at The University of Liverpool and Research Department at The Cardiothoracic Centre, Liverpool for his guidance and support throughout these studies. He helped culture the Radial Artery Smooth Muscle Cells when I was away or busy doing other experiments, completed the final few organ bath experiments in chapters 3 and 5. His help with the statistics and preparation of the paper that was based on the experimental work in chapter 5 was extremely helpful.

Abbreviations

5-HT - Serotonin

ACE – angiotensin converting enzyme

ATP – Adenosine Triphosphate

AVP - Arginine vasopressin

BIMA – Bilateral Internal Mammary Arteries

BSA – Bovine Serum Albumin

CABG – Coronary Artery Bypass Graft Surgery

CaM - Calmodulin

cAMP – Cyclic Adenosine Monphosphate

cGMP – Cyclic Guanosine Monphosphate

CT – Computerised Tomography

CVA – Cerebo-vascular Accident

DMEM – Dulbecco's Modified Eagle Medium

ET1 – Endothelin 1

FBS - Foetal Bovine Serum

FITC - flourescein isothiocyanate

GEA – Gastroepiploic Artery

HBS - HEPES-Buffered Saline

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IEA – Inferior Epigastric Artery

IMA – Internal Mammary Artery

K_{ATP} - ATP-sensitive potassium channels

KCO – Potassium Channel Opener

LAD – Left Anterior Descending Coronary Artery

LIMA – Left Internal Mammary Artery

MAO - monoamine oxidase

MI – Myocardial Infarction

MLCK – Myosin Light Chain Kinase

mRNA – Messenger Ribose Nucleic Acid

NA - Noradrenaline

NO – Nitric Oxide

PBS - Phosphate Buffered Saline

PhB –Phenoxybenzamine

RA – Radial Artery

RASMC – Radial Artery Smooth Muscle Cells

SpO_2 - Peripheral Oxygen Saturation

TIA – Transient Ischemic Attack

Contents

Chapter 1

General Introduction

Introduction

Coronary artery bypass graft surgery (CABG) is one of the most common operations performed in the western world. In the UK over 25,000 patients undergo CABG a year (1). It is a very successful operation with an in-hospital 30 day mortality rate of less than 2% (2). Over 70% of patients are free from angina and a further 25% report an improvement in symptoms (2). One of the problems with CABG is the recurrence of angina 5 to 10 years after the operation. This occurs due to narrowing of the conduit (typically the long saphenous vein), due to intimal hyperplasia, and has led surgeons to use arterial conduits which are not as prone to failure in this way.

1.1 History of Radial Artery as a Conduit in Coronary Artery Bypass Surgery

1.1.1 Coronary Artery Bypass Surgery

The first attempt to revascularise the heart using an artery was described by Vineberg in 1964. He described 140 operations where he mobilised the left internal mammary artery (LIMA), ligated the artery distally, divided it, and implanted the bleeding end into a tunnel in the left ventricular muscle close to the left anterior descending coronary artery (LAD) (3). At a similar time Gordon Murray reported resecting a diseased part of the LAD and replacing it with a vascular graft (4). Coronary endarterectomy (excision of atheroma) or surgical excision was the operation of choice in the late 1950's before coronary angiography and cardiopulmonary bypass. Longmire reported the first direct anastomosis between the LIMA and the right coronary artery.

"At the time we were doing the coronary thromboendarterectomy procedure and we also performed a couple of the earliest internal mammary to coronary anastomosis. We were forced into it when the coronary artery we were endarterectomising disintegrated and in desperation we anastomosed the left internal mammary artery to the distal end of the right coronary artery and later decided it was a good operation" (5).

With the development of direct coronary angiography in the early 1960's, surgeons were able to determine exactly where the narrowing in the coronary artery causing the patients angina was. It was only a matter of time before surgeons bypassed diseased parts of the coronary arteries to achieve normal flow. In 1963, Sabiston first performed an aorto-coronary saphenous vein graft, unfortunately the patient died 3 days later of a cerebral complication (6). Edward Garrett probably performed the first successful saphenous vein graft in 1964 in order to wean a patient from bypass (6).

The work of Rene Favaloro at the Cleveland Clinic in 1967 launched the widespread application of coronary artery surgery. In 1968 he published a series of 15 patients with no mortality. At the time of publishing the number of patients had risen to 55 with a less than 2% mortality (7).

1.1.2 Arterial Grafts

Coronary artery surgery widely became established with the long saphenous vein being the conduit of choice. It was the work of George Green (8) that led to some surgeons

using the LIMA as a conduit. In the early 1980's the greater long-term patency of the LIMA over the long saphenous became apparent. In 1986 Loop proved better patient survival at 10 years when the LIMA was anastomosed to the LAD rather than the long saphenous vein in patients with single, double and triple vessel disease (9). This was also echoed in the results of other groups (10;11), and its use as a conduit became widespread. Not only does the use of a single IMA increase survival it reduces the incidence of recurrent angina, late myocardial infarction and the need for further cardiac interventions (9;12). Now-a-days use of the LIMA occurs in over 90% of patients with disease in the LAD.

The survival benefit of the IMA graft is almost certainly due to its resistance to atherosclerosis (13). In most studies, early IMA patency is 95%-100% (14;15), but more importantly the 10 year patency is 95% compared to the 25-50% patency of the long saphenous vein (16).

With the superiority of both free and pedicled LIMA grafts over the long saphenous vein well established, surgeons began using both IMA's and the search for other arterial grafts was on. Additional arterial grafts used are the radial artery (RA), gastroepiploic artery (GEA), inferior epigastric artery (IEA), subscapular artery, lateral femoral circumflex artery, inferior mesenteric artery, and ulnar artery.

Lytle et al have now shown that two IMA's are better than one in terms of increased survival and fewer cardiac events (17). This has been confirmed by other authors (18-

20). A meta-analysis of almost 16000 patients performed by Taggart *et al.* found a survival benefit at 10 years in patients with bilateral internal mammary arteries (BIMA) compared to those with a single IMA (hazard ratio for death 0.81; 95% CI 0.70-0.94) (13). The results of this meta-analysis have to be interpreted cautiously however as the data was from non-randomised trials

One of the perceived problems with BIMA grafting is the increased perioperative morbidity and mortality, especially the risk of sternal wound infections, and respiratory morbidity. There is evidence from several large institutions that BIMA grafting does not increase perioperative mortality in appropriately chosen patients and with surgeons experienced in the technique (14;17;19-21). In terms of morbidity sternal dehiscence is the most worrying complication, especially in diabetics. Most studies report an increase in incidence of sternal wound infection following the use of BIMA (22;23) however others claim that suggestions that BIMA increases pulmonary morbidity are unfounded (24).

Despite evidence of clinical and survival benefits of using more than one arterial graft (25) the absence of any large randomised trials with long term follow-up, along with the increased technical demands of multiple arterial conduits, has precluded widespread use. In the UK only 15% of patients undergoing CABG receive more than one arterial graft (26)

Gastroepiploic Artery

The right gastroepiploic artery (GEA) was first used in myocardial revascularisation by Bailey and associates in a Vineberg-type myocardial implantation (27). 1n 1974 W Sterling Edwards used the GEA as a direct bypass graft to the right coronary artery (28). The first series of patients using the GEA as a conduit for CABG was described in 1987 (29;30). The GEA has reported short to midterm patency rates of 80%-97% (28;31). One of the advantages of the right GEA is that it can be used as a pedicled or free graft.

Inferior Epigastric Artery

The inferior epigastric artery (IEA) is an anatomical continuation of the IMA. It has a similar internal diameter and histological structure to that of the IMA (32). The use of the IEA as a conduit for CABG was reported by Puig in 1988 (33). He later went on to publish a series of 22 patients who underwent CABG with the IEA (32). Of the seventeen patients who underwent early angiography the patency rate was 88%. The two grafts that were blocked were anastomosed directly to the aorta. The size mismatch between the aortic wall and the diameter of the IEA may explain why these two grafts were blocked and an anastomosis onto a pericardial or vein patch may be better. Another way of avoiding an aorto-IEA anastomosis is to perform composite arterial grafting (anastomosing IEA to another graft). Calafiore has achieved excellent patency rates using the IEA this way (34). Midterm patency rates of around 90% have now been reported for the IEA (35).

The main advantages of the IEA are its ease of harvest and its similarities with the IMA. The disadvantages of using it are the postoperative pain a patient will get after an incision in the abdominal wall. This leads to a decreased willingness to cough, predisposing them to chest infections. The size mismatch with the aortic wall is another concern which does not occur with the RA which has a wider lumen and thicker wall.

1.1.3 The Radial Artery

The use of the RA as an alternative conduit for CABG was first reported by Carpentier in 1973 (36). Two years later, he recommended the technique should be abandoned because of a 35% incidence of narrowing or occlusion of this conduit at control arteriography (Discussion of Carpentier A (37)). These poor results were echoed by others (38;39).

Carpentier thought the initial failure was due to spasm of the dennervated artery (Discussion of Carpentier A (37)). Others suggested it was due to intimal hyperplasia (38).

In 1987 Carpentier received a referral in which the recent angiogram showed a RA that was occluded immediately after the original operation and was now patent with no visible atherosclerosis 15 years after the original operation. This prompted him to reinvestigate two further patients who had blocked radial arteries initially; both of these were now patent and disease free (40). These results and the availability of new antispasmodic drugs stimulated Carpentiers' group to reinvestigate the RA

His group reported a series of 104 patients who underwent myocardial revascularisation using a RA with excellent results (40). The one year patency was 93.5% (40). Their improved results were attributed to a more meticulous harvest technique and the use of antispasmodic agents. The RA was harvested as a pedicle and metal probes as a means of vasodilatation were avoided. Topical papaverine was used to reverse any spasm intraoperatively and diltiazem was used as an antispasmodic in the postoperative period.

These changes in the methods of harvesting the RA probably lead to better preservation of the endothelium resulting from decreased handling and the lack of probing of the artery. There would also have been a decreased incidence of spasm due to bathing the graft in a vasodilator solution and the use of calcium channel blockers (41). The midterm results of RA grafts are very promising (see table 1.1).

Author	Immediate Patency	1 year Patency	5 year Patency	10 year Patency	Method
Brodman *e* (42)	95.7%				Elective angiography
Possati GF		93.1%	92%(43)	91.6% (44)	Elective angiography
Acar	99% (40)	92% (40)	83% (45)		Angiography of consecutive patients
Buxton RAPCO trial (46)			95%(less than 70 years old) 86%>70 years old)		Elective angiography
Tatoulis (47)		90.2% (mean 14.4 months)			Mainly symptomatic angiography
Iaco AL (48)	98.9%		95.6%		Voluntary angiograms
Da Costa (49)	96%	100%			Elective angiography

Table 1.1 Reported Patency of Rates Radial Artery at Various Time Intervals and Method Used to Measure Patency

The RA is a versatile conduit that can be harvested easily and safely, and has handling characteristics superior to that of other arterial grafts (50). It is long enough to comfortably reach any coronary artery from the ascending aorta. The size of the proximal end of the RA is bigger than that of other arterial conduits, such as the IMA and IEA, this makes a proximal anastomosis to the wall of the ascending aorta easier and one does not have to worry as much about a size mismatch.

The RA has a great ability to adapt its diameter to the flow necessary (flow mediated vasodilatation and vasoconstriction). In the presence of a large run off (occluded or tightly stenosed coronary artery supplying a large territory) the artery dilates. However the reverse happens in areas of low flow, the RA contracts reducing its internal diameter: this behaviour is evident angiographically, the so called string sign. This phenomenon has led some authors to recommend that the RA should only be used as a conduit to graft arteries with a high run off (34).

Use of the RA does not increase the complexity or morbidity of CABG (51). Its harvest is associated with minimal significant morbidity. Although up to 67% of patients complain of some sensory symptoms related to RA harvest, no patients said these symptoms interfered with daily activities (52). Patients report sensation loss far more often than it can be objectively measured. Royse *et al.* report only 2.1% of patients having detectable sensory loss in the distribution of the lateral cutaneous nerve of the forearm and 0.3% in the distribution of the superficial branch of the radial nerve (53).

The differences are probably due to patients perceiving scar tightness or hypersensitivity as sensory symptoms.

Patients rarely complain of any functional impairment of the arm following RA harvest. Saeed *et al.* quote an incidence of 3% of patients complaining of mild limitation in hand activity (52). Functional assessment of the arm following RA harvest reveals only slight decrease in strength or sensory loss. Royse *et al.* found grip strength to be 4.8% less and finger pinch strength to be 5.1% less when compared with the non-operated arm (53). One has to remember that it was usually the non-dominant arm that was used for RA harvest and these small differences may merely reflect the difference between the two arms. The incidence of wound infection rate following RA harvest is between 0 and 6 % (52). Numerous studies have shown this to be less than that following saphenous vein harvest (52).

One of the most feared complications of RA harvest is hand ischaemia (54). This however is extremely rare. Out of 6,646 patients having at least one RA harvested Tatoulis *et al.* report only two patients with finger tip ischaemia (47). Both of these patients had scleroderma and Raynaud's phenomenon, which many surgeons would say was a contra-indication to RA harvest.

Preoperative and postoperative index finger pulse oximetry reveals an increase in saturation following RA harvest and CABG, both in operated and non-operated index fingers (53). This probably indicates the SpO_2 for the whole patient increased following

surgery. There is certainly no decrease in oxygen saturation in the hand following RA harvest. This correlates with the extremely low incidence of hand ischaemia.

Although the results of the RA as a bypass graft are excellent, the RA has one main drawback, that of spasm. Spasm is the spontaneous contraction of the artery. It can lead to myocardial hypoperfusion post-operatively leading to perioperative myocardial infarction (MI) and death. The RA is classified as a type III functional artery (55). This type of artery is more reactive to vasoconstrictors than somatic arteries located in the body wall (type 1, such as IMA and IEA) or splanchnic arteries (type II e.g. GEA). Spasm occurs frequently during harvesting of the RA so that pharmacological agents (vasodilators) are required intraoperatively to relieve this prior to grafting. These can be applied topically to the RA once harvested, prior to grafting. Spasm may also occur in the postoperative period. Cable *et al.* quote an incidence of spasm in 5 to 10% of RA grafts postoperatively (56). Spasm in the postoperative period must be treated with intravenous or oral vasodilators or be prevented by the use of long acting topical pharmacological agents at the time of surgery. The reported incidences of spasm are shown in table 1.2.

Group	Incidence	Time Following Surgery
Acar (40)	4-5%	First three weeks
Manasse (57)	10%	Mean 24.3 days
Iaco (48)	0%	Mean 18 days
Calafiore (34)	1.3%	Within 12 months of op
Da Costa (49)	10%	On hospital discharge
Chen (58)	4.3%	Mean 11.6 weeks

Table 1.2 Reported Incidence of Spasm and Interval Following Surgery Measured

1.2 Preoperative Assessment of the Hand Circulation

The Allen's test is the most commonly used test to assess the collateral circulation prior to RA harvest. The ulnar and radial arteries are compressed at the wrist for 30 seconds to induce hand ischaemia. Blood is evacuated from the hand by clenching the fist. The ulnar artery is released and if hyperaemic reperfusion (change in colour from white to red) at the tips of the thumb and index finger occurs in 5 seconds or less the test is called normal and the RA harvested. If reperfusion occurs in 6-10 seconds then the test is called equivocal and mostly the RA is used. If it takes longer than 10 seconds then the test is deemed abnormal and the RA not used.

Other methods used to assess the hand circulation include doppler studies and digital plethysmography (59).

1.3 Harvesting of the Radial Artery

The arm and chest are prepared and draped together. The arm is placed on an arm board attached to the operating table so that no traction on the arm occurs when the table height is varied.

The incision extends from 1cm medial and distal to the biceps tendon at the elbow, to 1cm medial and proximal to the radial styloid process. It should take the shape of 'a lazy S'. Diathermy is used to divide the tissues to the deep fascia. Care is taken to avoid the lateral cutaneous nerve of the forearm which crosses the RA from lateral to medial near the distal extremity of the incision. The deep fascia is then divided along the medial edge of the brachioradialis muscle.

Deep to the brachioradialis muscle lays a well defined fascia surrounding the RA and its venae commitantes. Once this fascia is divided the RA lies within loose areolar tissue and is very easily harvested. The deep fascia and the fascia surrounding the RA fuse in the distal third of the forearm and should be divided together. The branches of the RA are then divided using a combination of diathermy and metal clips. Some people prefer to avoid the use of diathermy as they feel this may cause damage to the RA. Others feel the use of metal clips increases the handling of the RA and hence spasm. The harmonic

scalpel may be used to harvest the RA and has been shown to reduce collateral damage (59;60). During harvesting neither the artery or the venae commitantes on either side of the RA should be grasped in order to prevent spasm.

The RA is divided proximally first, distal to the origin of the ulnar artery. If the full length of the artery is not required then the large muscular branch to brachioradialis can be preserved. Once divided proximally, one checks for back flow from the divided proximal end of the RA to confirm adequate collateral circulation, the squirt test (61). The artery is then divided distally and stored ready for grafting.

The proximal and distal stumps are ligated and transfixed. The fat and skin, but not the deep fascia is closed. The arm is then bandaged and placed by the patients' side and the operation continues.

1.4 Morphology, Physiology and Pharmacology of the Radial Artery

The forearm and hand receives its blood supply from the radial and ulnar arteries. The RA commences at the bifurcation of the brachial artery, just below the elbow. It passes along the radial side of the forearm to the wrist. It then winds backward, around the lateral side of the carpus, beneath the tendons of the Abductor pollicis longus and Extensor pollicis longus and brevis to the upper end of the space between the metacarpal bones of the thumb and index finger. Finally it passes forward between the two heads of the first interosseous dorsalis, into the palm of the hand, where it crosses the metacarpal

bones and at the ulnar side of the hand it unites with the deep palmar branch of the ulnar artery to form the deep palmar arch.

There are extensive anastomoses between the branches of the radial and ulnar arteries. The superficial and deep palmar arches are the most important of these circuits because they provide the blood supply to all of the fingers (62). It is these anastomoses that allow the hand to still receive an adequate blood supply following harvest of the RA.

Variations in the blood supply to the hand are common. The deep palmar arch is incomplete in 10% of hands and the superficial palmar arch incomplete in 34% of hands (62). Ruengsakulrach *et al.* in the 50 hands they studied found no cases of both an incomplete deep and superficial palmar arches (62). From an anatomical point of view it is therefore safe to remove the RA from its origin proximally, and to the level of the wrist distally. This is backed up in the literature as reports of hand ischaemia are rare (54). In the Melbourne experience of over 6000 RA's any hand ischaemia that occurred was in patients with vasomotor disorders (47).

When one looks at the biological characteristics of any artery it is helpful to divide it up into the structure and function of the endothelium and that of the smooth muscle. One must remember though that these two components do not act independently.

The RA is a very muscular artery that has a media rich in leiomyocyte (63). In the RA myocytes within the media are organised in tight layers, whereas in the IMA there is irregular organisation of myocytes with a loose structure of connective tissue and elastic fibres (56).

The vasa vasorum of the RA do not penetrate into the media, oxygen and nutrients are provided by diffusion (63). This suggests that transposition of the artery as a free graft should not have adverse ischaemic implications.

Regional variations occur in both the functional and morphological characteristics of the RA (64). This regional variation should be taken into consideration when choosing a section of RA for a bypass graft. There is more smooth muscle in the wall of the proximal RA than that of the distal RA, This is reflected in the greater contractility of the proximal RA to KCl (64). Despite the increased contractility of the proximal RA it is usually the distal end that goes into spasm in clinical practice (65). The explanation for this could be that any contraction of the RA has a greater effect on the smaller distal end.

The reason for superior performance of arterial grafts is not fully understood. The IMA seems to be free of the intimal thickening noted in coronary arteries with advancing age (66;67). The preserved and perfused vasa vasora of the IMA graft may be a factor that contributes to the increased patency. Another factor that may contribute to the long-term patency of the IMA graft is the endothelial function (68). Prostacyclin production

from the IMA endothelium, when compared to that of the saphenous vein is greater (69). He and Yang could not detect significant differences in endothelial function between the RA and IMA (68).

It is well reported that saphenous vein grafts are prone to the development of intimal hyperplasia, followed by atheromatous change. This characteristic has been demonstrated histologically and is responsible for the poor long-term results of this conduit (70;71). Hagiwara *et al.* describes a method to study coronary artery bypass grafts *in vivo* using intravascular ultrasound imaging. They studied 15 RA grafts and found no change in thickness of the intima or the media in the mid-term (70). With such small numbers definitive conclusions regarding patency or comparisons to other grafts are difficult to make and the authors' conclusion that structural changes rarely developed in RA grafts in the early years after surgery is speculative.

Various vasoconstrictors have been suggested to be the cause of spasm in human arterial grafts (72). Studies have shown that the RA has a greater contractile response than the IMA to vasoconstrictors including noradrenaline (NA), endothelin 1 (ET 1), serotonin (5-HT), and angiotensin I and II (68;73-75). He and Yang demonstrated that the contractile force to KCl, normalised for circumference, was similar in the RA and IMA (68), However, even when one has normalised for vessel wall thickness, NA, vasopressin, angiotensin II and ET 1 produce greater increases in tension compared to the IMA, suggesting that the increased contractility in the RA is receptor mediated (68).

To support this their study showed that there was no increase in sensitivity of the RA compared with the IMA to ET1 and angiotensin II (68).

For these reasons it is obviously important that we need a detailed knowledge of the characteristics of the receptors in the RA. The human RA is an α_1-adrenoceptor dominant artery with little β–adrenoceptor function (76). This absence of β–adrenoceptor function differs from that of the IMA (77). Also in the RA α_2-adrenoceptors are present (76). β–adrenoceptors mediate relaxation of large arteries. They tend to contract when β–blockers are given because the β–mediated relaxation is eliminated as reported in coronary arteries (78). The absence of β–adrenoceptors clearly demonstrate that β–blockers will not cause spasm in the RA.

Post-junctional α-adrenoceptors are composed of α_1 and α_2 subtypes. The contribution of α_2-adrenoceptor to drug induced contraction depends on particular blood vessels and species, as well as the diameter of the blood vessel (79;80). In the RA, drug induced contraction is via both α_1 and α_2 subtypes (76). It is unknown which subtype of adrenoceptors mediate neuronal sympathetic vasoconstriction in the human RA. Medgett and Langer (81) reported that α_2-adrenoceptors do not mediate neuronal sympathetic vasoconstriction in the cat middle cerebral artery as they are located extra-junctionally. However more recent reports have shown that postsynaptic α_2-adrenoceptor can be involved in the vasoconstrictor response to sympathetic nerve stimulation to a significant (82) or even predominant level (76;83).

Other receptors present on the RA that may contribute to spasm include the 5-HT_2 receptor for 5-HT, endothelin A receptor for ET I (84) and the AT-1 receptor which mediates angiotensin II induce contractions (73).

1.5 Mechanisms of spasm

The true incidence of vasospasm in the human RA is difficult to determine as many cases may go undetected (85;86). Reports of perioperative ischaemia in patients with RA bypass grafts (87) and the observed reduction in postoperative markers of MI in patients receiving RA grafts treated with PhB as compared with those treated with verapamil/ glyceryl trinitrate (88), would support the need for effective, postoperative vasodilator therapy.

Below is the CT scan of a patient who was being followed up for mediastinal lymphadenopathy that had been found on a pre-operative CT scan. At 6 weeks post-operative no flow in the RA can be identified, its position being identified by surgiclips (figure 1.1a). However at 3 months postoperative good flow is seen in the graft (figure 1.1b).

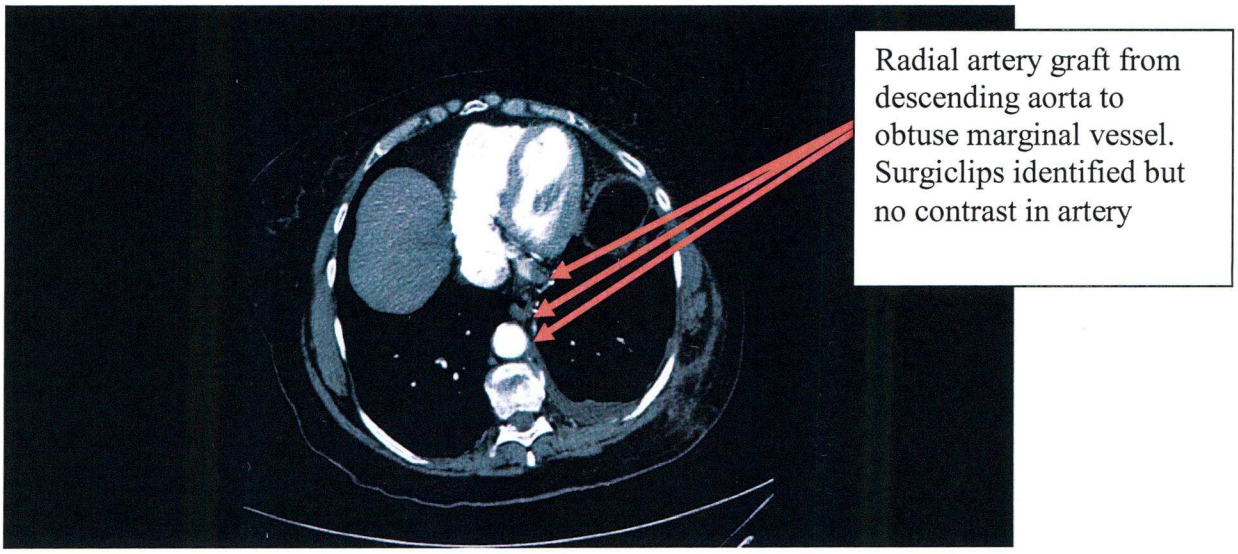

Figure 1a – The radial artery can be identified by the position of the surgiclips, but no contrast is present in the artery i.e. the artery is blocked or in spasm.

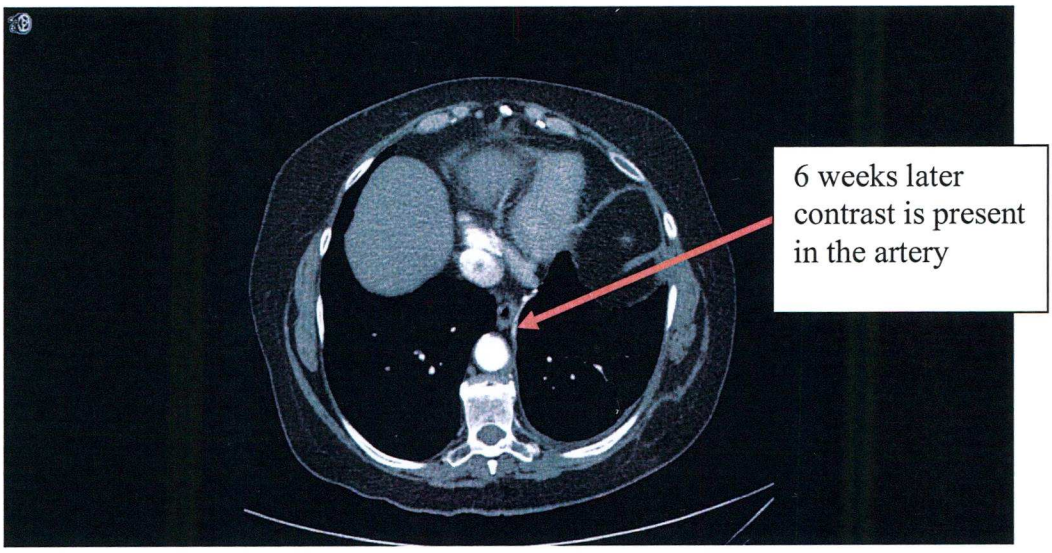

Figure 1b – Contrast is now present in the radial artery showing that it was in spasm, not blocked 6 weeks earlier.

Figure 1.1 - Transaxial views of CT scan of the chest of a patient who had redo coronary artery surgery. A RA was anastomosed from the descending aorta to the 1st obtuse marginal vessel. A CT scan performed pre-operatively, to assess the position of the internal artery behind the sternum, had identified enlarged pre-tracheal lymph nodes. These were followed up on interval CT scans at 6 weeks (figure 1a) and 12 weeks post-operatively (figure 1b).

Spasm can occur both during harvesting and after the graft is connected, the aetiology is likely to be multi-factorial. Factors probably involved in the mechanism of spasm include surgical trauma, locally released vasoconstrictors, neural factors, and circulating hormones

One of the problems about trying to understand the mechanisms of spasm is that isolated tissue assays can only determine what factors have the potential to cause spasm not the cause of it *in vivo*.

The most likely cause of spasm at the time of harvesting is surgical trauma. Smooth muscle in the wall of the RA will contract in response to mechanical stimulation during harvesting and grafting. It was changes in the harvesting technique along with the use of calcium channel antagonists that led to the revival of the RA as a conduit for CABG (40). Surgical trauma can be minimised by harvesting the artery as a pedicle (along with accompanying veins and areolar tissue) rather than skeletonising it. Taggart *et al.* has described successfully using the RA after skeletonising it. This is possible if meticulous surgical technique is used and vasodilators used to reverse any spasm prior to grafting (89). However, we feel that spasm is best managed by prevention, rather than treatment after it has occurred, and therefore do not advocate the use of this technique. Another cause of spasm in the perioperative period may be the temperature changes the RA may experience (90). The RA should be stored in a solution at 37°C after harvesting, prior to grafting, to prevent any temperature induced spasm.

The aetiology of the cause of spasm in the postoperative period is more difficult to determine. Undoubtedly, levels of vasoconstrictors are raised in the postoperative period. These vasoconstrictors include NA, adrenaline, angiotensin II, vasopressin, ET I, thromboxane A_2, prostaglandin $F_{2\alpha}$ and 5-HT (91). Any or all of these factors may be responsible for spasm. One of the problems with this theory of spasm is that although raised levels of these vasoconstrictors are present in the postoperative period they reach nowhere near the EC_{50} for these compounds *in vitro* (see table 1.3), and also the levels of these compounds return to normal within a few days after surgery, spasm may still occur after this time.

Vasoconstrictor	**Post-operative plasma level**	**EC_{50} in RA**
Endothelin 1	0.086nM (74)	117nM (68)
Angiotensin II	36pM (74)	3.5-6.9nM (68;73)
Noradrenaline	0.17μM (74)	1.8μM (92)
Vasopressin	100pg/ml (93)	1.9nM (94)

Table 1.3 – Comparisons of *in vivo* levels of vasoconstrictors following CABG and *the in vitro* EC_{50} of the vasoconstrictor on the human RA

Although the plasma levels of these vasoconstrictors do not reach levels that would cause a contraction *in vitro*, local levels in vivo may be higher than plasma levels. ET I is a potent long acting, calcium dependent vasoconstrictor produced by endothelial cells (95). Although small amounts are present in the plasma it acts locally (91). Endothelial cells are activated during CABG by mechanical and chemical stimuli such as thrombin, bradykinin and adrenaline. Activation leads to increased ET I production (96).

Thromboxane A_2 is derived from platelets. Platelets are activated during cardiopulmonary bypass leading to an increased production and release of thromboxane A_2 (91). Platelets may adhere to any damaged endothelium in the RA releasing thromboxane A_2 at high local concentrations.

As well as endogenous vasoconstrictors, endogenous vasodilators such as nitric oxide and natriuretic peptide are released after CABG. Therefore, exogenous vasoconstrictors are often required to maintain the patients' blood pressure postoperatively. Commonly used vasoconstrictors include NA and phenylephrine. Vasopressin is becoming more commonly used as a vasoconstrictor following cardiac surgery.

Vasopressin is synthesised in the magnocellular nuclei of the hypothalamus. It is then axonally transported to the neurohypophysis where it is released into the blood. Vasopressin is a critical controller of body water balance. Its primary action is to increase water reabsorption by the kidney. Its output is primarily regulated by the electrical activity of the magnocellular neurones. During dehydration the firing of these

neurones is increased via activation of peripheral, central and intrinsic osmoreceptors sensing a rise in plasma osmolarity (97).

Arginine vasopressin (AVP) has little vasoconstrictor effect in haemodynamically normal subjects but is a potent vasoconstrictor in states associated with arterial hypotension (98;99). AVP is one of the most potent coronary artery vasoconstrictors known (100). AVP has a great propensity to cause vasospasm in coronary bypass conduits (101). This occurs as a direct effect evoking contraction of the vascular smooth muscle via the V1 receptor (102) and also by facilitating sympathetic neurotransmission and promotes the constrictor effects of NA (103).

Vasodilatory shock induced by cardiopulmonary bypass, is usually mild, and requires low doses of vasopressor support to maintain perfusion for a few hours postoperatively. However in 8% of cases, a more severe state of shock develops necessitating high dose vasopressor therapy (104). The administration of high dose catecholamine vasopressors is associated with complications related to end organ perfusion and their effectiveness is limited by catecholamine resistance (105). Vasopressin levels have been shown to increase more than 6 times following CABG, and can reach levels of 100pg/ml (93). Patients with vasodilatory shock following CABG, although having AVP levels within the normal range, have been shown to have inappropriately low levels of AVP for the degree of hypotension present (104). These findings have led to some authors recommending the use of exogenous vasopressin to treat vasodilatory shock following cardio-pulmonary bypass (104).

Plasma NA levels are raised in the postoperative period (91). The NA is derived from sympathetic nerves. The RA has a dense network of sympathetic nerves in its wall. In this thesis I will investigate whether degenerating nerve endings in the wall of the RA have the potential to release sufficient NA to cause spasm.

Noradrenergic Transmission

Synthesis of NA

The starting material is dietary L-phenylalanine. This is absorbed in the small intestine and oxidized by hepatic phenylalanine hydroxylase to form L-tyrosine. This is circulated in the bloodstream and actively taken up into the cytoplasm of noradrenergic nerves. Here it is converted to dopamine. Dopamine is actively transported into the transmitter storage vesicles. Here it is oxidised to NA.

Storage of NA in vesicles

Endogenous NA is stored in vesicles which are formed in the cell body and transported to the axon terminals by axoplasmic flow. Within the vesicle storage of NA is aided by the presence of adenosine triphosphate (ATP) (forms a weak complex), a sulphomuccopolysaccharide and a soluble protein called chromogranin. The resistance to diffusion is provided by the vesicular contents and the continued operation of the amine uptake process in the vesicle membrane.

Release of NA from Sympathetic Nerve Endings *in vitro*

NA can be released from nerve endings by electrical stimulation or by chemical treatments which either destroy the nerve terminal itself or displace the stored NA. Tyramine is a compound with a similar chemical structure to NA. (Figures 1.2 and 1.3) It has lost the catechol-OH group and the β–OH group.

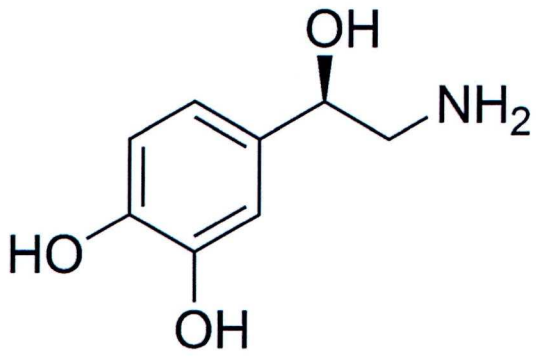

Figure 1.2 - Chemical structure of Noradrenaline

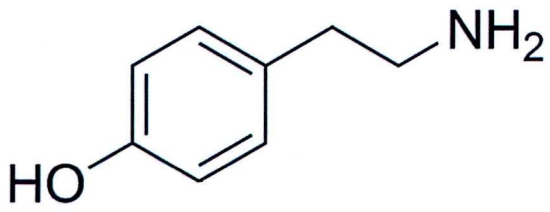

Figure 1.3 - Chemical structure of Tyramine

Tyramine displaces NA from its storage vesicle in sympathetic nerve endings. We will be stimulating human RA rings with tyramine to see if it elicits a contractile response.

Synergism of vasoconstrictor responses

Despite the levels of vasoconstrictors in the plasma being insufficiently raised to cause a contraction of the RA *in vitro* alone, it has been shown that there is a synergistic effect between some vasoconstrictors e.g. NA and vasopressin. It may therefore be a combination of vasoconstrictors that are the cause of spasm *in vivo*. This will be briefly investigated in the appendix of this thesis.

1.6 Prevention and Treatment of Spasm

Various different drugs have been used to prevent and treat spasm. Pharmacological strategies to prevent spasm usually consist of an intra-operative strategy and a postoperative strategy. The intra-operative strategy is used to treat any spasm that occurs during the harvesting of the RA. Drugs are applied topically to the RA prior to grafting. Since these drugs do not enter the systemic circulation they can be used in high concentrations. Postoperative strategies are used to prevent spasm after the operation. They generally consist of giving a vasodilator via intravenous infusion until the patient can take oral medication. The length of time a patient should take antispasmodic medication is controversial. Some commonly used strategies are shown in table 1.4.

More recently PhB, an irreversible α adrenoceptor antagonist has been used to prevent spasm. This offers a novel approach to prevent spasm because the RA is treated prior to grafting and the effects of the drug should still be present in the postoperative period. In this thesis I will investigate the duration of action of PhB and look at other irreversible drugs that might have a role to play in the prevention and treatment of spasm.

Group	Operative Strategy	Post-operative Strategy
Acar(45)	Blood and papaverine	Intravenous diltiazem (0.1mg/kg/h) then oral (250mg daily)
Cable(56)	Lactated Ringer's, papaverine and verapamil	
Buxton (46)	Papaverine (1mM) in 50% blood, 50% ringers lactate	Intravenous milrinone (loading dose 25μg/Kg then 0.25μg/Kg/min for 12-18 hours) then oral amlodipine (2.5-5mg)
Calaforie(34)	Papaverine 1mg/ml in normal saline	Intravenous diltiazem (4mg/h) then oral diltiazem (60mg tds for 1 month)
Dietl and Benoit(65)	Papaverine and diltiazem if spasm noted	Diltiazem (0.1mg/Kg/h for 48hours them 120mg-240mg oral for 6-12 months)
Reyes(106)	Papaverine 1mg/ml blood	Diltiazem (loading dose is now 0.05 mg/kg followed by a continuous infusion of 0.15 to 0.2 μg / kg^{-1} / min)
He and Yang(107)	Verapamil (30μM) nitroglycerin (30μM) solution	Intravenous verapamil (0.5mg/h then 240mg oral daily for 1 year)

Table 1.4 Different Strategies Currently Used To Prevent Spasm. In some incidences no doses are given in the table because doses were not stated in the referenced paper

1.6.1 Glyceryl Trinitrate

This is a lipid soluble drug that penetrates cell membranes delivering the nitrite ion and free radical nitric oxide (NO) into the cytoplasm. NO inhibits receptor stimulated calcium release from intracellular stores and reduces calcium influx by hyperpolarising the cell membrane or inhibiting voltage gated calcium channels (86;108). In addition, NO promotes the reuptake of calcium into stores and calcium extrusion, as well as accelerating myosin light chain phosphorylation (86;109). This effectively means that GTN opposes both agonist mediated contraction and subsequent sensitisation caused by the activation of rho-kinase, as NO inhibits Rho A activation.

GTN may be applied topically to the RA prior to grafting to relieve any spasm present following harvesting, given intravenously in the perioperative period to prevent spasm, or orally for a period of time following the operation to prevent late spasm.
Average therapeutic plasma concentrations of GTN are $2x10^{-8}$ M (15-20μg/min intravenously) (110).

Both GTN and isosorbide dinitrate have been shown to significantly reduce KCl and NA mediated contraction of the RA (56). Isosorbide dinitrate was not as effective as GTN at reversing established KCl induced contractions of the RA (56).

Chanda *et al.* found GTN ($2x10^{-8}$ M) was equally effective at reversing spasm due to a combination of vasoconstrictors when compared to a combination of GTN ($1x10^{-8}$ M) and a calcium channel blocker (74).

GTN has been shown to be superior to diltiazem at causing vasodilatation of the RA both *in vitro* and *in vivo* (111;112). GTN is well tolerated in the postoperative period. Shapira *et al.* found that all patients in their trial randomised to receive intravenous GTN (n=84) were able to tolerate it, as were the 5 patients that crossed over from the intravenous diltiazem group because of side effects (112). Despite finding GTN to be better tolerated than diltiazem, and to be better at causing relaxation of the RA *in vitro* and *in vivo,* Shapira failed to show any clinical differences, such as mortality, perioperative MI, creatinine kinase MB isoenzyme level, or abnormal perfusion to the RA territory on thallium-221 stress testing, between patients receiving GTN and diltiazem that could be relate to spasm (112) The reason for this is presumably the relatively small numbers in their study.

One of the problems with GTN is that the vasodilatory effects are rapidly blunted because of the development of nitrate tolerance (113;114). Long-term treatment has been shown to be associated with cross tolerance to other endothelium-dependent vasodilators such as acetylcholine (115). Cross tolerance is probably due to an increased production of reactive oxygen species leading to an enhanced breakdown of endothelial-derived nitric oxide (116). Protein kinase C has been shown to be activated in patients

on GTN. This may suggest a potential role for protein kinase C inhibitors in preventing the development of nitrate tolerance and cross-tolerance.

Another one of the major problems with GTN is its relatively short duration of action. Mussa *et al.* found that verapamil/GTN solution was ineffective after 5 hours (117).

1.6.2 Calcium Channel Blockers

These drugs all act by binding to the α_{1c} subunit of the L-type calcium channel, which is the main pore forming unit of the channel (118). The L-type calcium channel is present in cardiac muscle, vascular smooth muscle, non-vascular smooth muscle and other tissues. They inhibit the influx of the Ca^{2+} into the cytoplasm. In vascular smooth muscle an increase in cytosolic Ca^{2+} leads to contraction. Therefore blockage of calcium entry into the cytoplasm will favour relaxation.

Calcium channel blockers are very effective in preventing or reducing the contraction to depolarising agents such as K^{+}. This is because calcium channel antagonists block calcium entry into the cell by blocking voltage operated calcium channels which is the major mechanism of the constricting action of depolarising agents. They are less effective at blocking contraction mediated via G-protien coupled membrane receptors such as vasopressin, angiotensin II, ET I and NA (41).

Many authors advocate the routine use of calcium channel blockers when the RA is used in CABG (34;40-42;65). The problem with these drugs is that they cause bradycardia,

hypotension due to a reduced cardiac output or rhythm disturbance and so their use is not advocated by others (119). Shapira *et al.* (120) found that 40% of patients receiving diltiazem post-operatively required temporary pacing, resulting in a longer intensive care unit stay. Diltiazem is also more expensive than GTN. 24 hours intravenous diltiazem is 10 times more expensive and 6 months oral diltiazem twice as much (112). There is also the expense of an increased intensive care stay and the expense of an increase in the incidence of temporary pacing to be taken into consideration.

The most effective group of calcium channel blockers at preventing spasm are the dihydropyridine group (121). It is the 1,4 dihydropyridine ring that probably gives them their increased vascular selectivity properties (122). Amlodipine has a more ionic charge than nifedipine making it more vascular selective, having less of an effect on myocardial contractility and a longer duration of action (122). These advantages may make amlodipine more suitable as an antispasmodic, especially in patients with an impaired left ventricular function, although nifedipine is a slightly better vasodilator *in vitro* (123). Of the various calcium channel blockers diltiazem is the least potent (124).

Cable *et al.* found that diltiazem and verapamil did not significantly alter the contraction of RA rings to KCl and NA, however nifedipine decreased the response to KCl and NA throughout the concentration response curve (56). Nifedipine was not as effective as GTN at reversing established KCl induced contractions of the RA (56). Angiographically provoked spasm by 5-HT infusion also suggests that diltiazem is ineffective at treating spasm (57).

Acar *et al.* recommend the long-term prescription of calcium channel blockers in patients receiving a RA graft (45). However in this study only 60% of patients continued with the calcium channel blocker and no difference in patency of the RA graft could be detected between the two groups (45). Possati (44) also found no difference in the patency of the RA at 10 years in patients who continued the calcium channel blockers beyond the first year.

Average therapeutic plasma concentrations

Nifedipine	0.28μM (125)
	(10-20mg orally 8 hourly or 5-15 μg/kg/min intravenously)
Diltiazem	560nM (125)
	(60-90mg orally 8 hourly or 75-150 μg/kg/min intravenously)
Verapamil	0.22 μM (125)
	(80-160mg orally 8 hourly or 150 μg/kg/min intravenously)
Nicardipine	5610 nM (126)
	(1 μg/kg/min intravenously)

.

Since calcium channel blockers provide vasoconstrictor-selective vasodilatation in human arterial grafts, He and Yang (41) proposed using a combination of verapamil and GTN. This combines the advantages of the highly selective blockade to voltage-dependent calcium channels and longer lasting effects of calcium channel blockers with the rapid onset, short acting non-specific effect of GTN. They had previously shown

this combination to be useful in other conduits used for CABG (127;128). They found that a solution of verapamil and nitroglycerin induced more rapid relaxation of the RA than papaverine (41) and later went on to show it was less harmful to the endothelium (107).

1.6.3 Phosphodiesterase Inhibitors

Phosphodiesterases are a family of isoenzymes that are responsible for the breakdown of phosphodiesters such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Type III phosphodiesterase inhibitors e.g. enoximone, milnirone or amrinone prevent the breakdown of cAMP. In cardiac muscle the increased intracellular concentrations of cAMP cause an increase in myocardial contractility. Increased cytoplasmic concentrations of cAMP in smooth muscle cells, leads to the reuptake of Ca^{2+} into the sarcoplasmic reticulum, and therefore relaxation. This ability to relax smooth muscle has led to the use of phosphodiesterase inhibitors to prevent spasm in the RA.

Papaverine is a smooth muscle relaxant whose mode of action is poorly understood It is believed to act predominantly as a phosphodiesterase inhibitor (129) although it also decreases calcium influx (130) and inhibits release of calcium from intracellular stores (131).

The problem with papaverine is that it has consistently been shown to damage the endothelium (132). Although this work has been mainly on the IMA and long saphenous vein one would expect similar damage to occur to the endothelium of the RA.

Dipp *et al.* showed that papaverine was more harmful to the endothelium of the RA than PhB (129). Papaverine has a duration of action of less than 1 hour (117). This damage to the endothelium and short duration of action limits its usefulness as an antispasmodic in RA conduits.

It has been shown that enoximone and milrinone are capable of causing nearly complete relaxation to the contractions produced by thromboxane A_2 mimetic U46619, NA or KCl in the IMA *in-vitro* (133;134). This effect of enoximone was potentiated by dobutamine (133). Liu *et al.* found milrinone to be more potent than papaverine but not as potent as GTN or sodium nitroprusside at preventing spasm (134). Any inhibitory effect of milrinone occurred as a decrease in the maximal response not as an increase in the EC_{50} indicating its effect is directly on the smooth muscle.

The therapeutic levels of enoximone are $7.3x10^{-6}$M. This is achieved with a bolus of 1000 μg/Kg followed by an intravenous infusion of 10μg/Kg/min (135). This level only caused 0-30% relaxation in IMA rings (133). To back the evidence up that enoximone may not be a useful vasodilator at therapeutic concentrations Cracowski *et al.* have previously shown no increase in IMA blood flow when the patient was given a bolus injection of 200μg/Kg enoximone (136).

In contrast to enoximone, milrinone may be a clinical relevant vasodilator on human IMA (137). Plasma concentrations of milrinone are EC_{50} of 167ng/ml (7μM) (138). Milrinone has the advantage over other antispasmodics of being positively inotropic.

Also as its action is endothelium independent, it may be useful to prevent spasm due to endothelial dysfunction. He and Yang have shown milrinone to be a potent vasodilator of the RA at therapeutic levels (139). They found milrinone caused an increase in the EC_{50} and a reduction in the maximal response to KCl, phenylephrine and U46619 (139). This differs from what has been shown in the IMA (134).

Sildenafil is a selective inhibitor of type5 cGMP phosphodiesterase. It is used to treat erectile dysfunction in men (140). Inhibition of cGMP phosphodiesterases leads to an increase in intracellular cGMP in vascular smooth muscle. This reduces intracellular calcium levels by stimulating Ca^{2+}-pumping adenosine triphosphatases, or by opening Ca^{2+}-activated K^+ channels (141). Sildenafil has been shown to cause significant relaxation in the human RA *in vitro* (142). The relaxation of the RA by the NO donor sodium nitroprusside was amplified in the presence of sildenafil (142). Type 5 phosphodiesterase is not present in the human myocardium, therefore sildenafil is not a positive inotrope unlike the type 3 phosphodiesterase inhibitors. Before its use clinically more studies are needed to assess its efficacy compared to other antispasmodics.

1.6.4 Phenoxybenzamine

Phenoxybenzamine (PhB) is a non-competitive antagonist of the α adrenoceptor. It is a β-haloalkylamine related to the nitrogen mustards. It acts by irreversibly alkylating the α adrenoceptor on the cell membrane.

Taggart *et al.* first described the uses of PhB as an antispasmodic (143). He later went on to show that its duration of action was greater than that of papaverine and less

harmful to the endothelium (129). It has also been shown that PhB was more effective and lasted longer than verapamil/nitroglycerin solution (117).

Velez *et al.* found that a much smaller dose of PhB was necessary to observe a maximal effect in canine radial arteries (144). They observed a maximal effect at a dose of 1μM compared to the 6mM that Taggart recommended using. Also they achieved this maximal effect after a 30 minutes treatment period compared with the one hour period described by Taggart (143). Corvera *et al.* observed a maximal effect at 1mM in human radial arteries with a 30 minute incubation period (145). This shorter treatment period is important as it is what happens in clinical practice.

NA is a mixed α and β adrenergic agonist. Antagonism of the α adrenoceptors not only prevents contraction of the RA due to NA, it unmasks the vasodilatation component of the β adrenoceptors. Stimulation of the β adrenoceptors by NA after α adrenoceptor blockade by PhB in the RA will lead to vasodilatation (145).

A similar thing is seen with another commonly used inotrope in cardiac surgery, dopamine. In the peripheral vasculature dopamine exerts its effects via the D_1 dopaminergic receptor and α adrenoceptors. Inhibition of the α adrenoceptor by PhB will unmask the D_1 effect of dopamine which will lead to a mild vasodilatation (146).

The duration of action of PhB in the human RA is unknown. Velez *et al.* found that the effects of PhB lasted 48 hours in canine RA rings that were stored in an incubator (144).

The lack of regeneration of α-adrenoceptors in this experiment may be due to the flaccid conditions the rings were incubated under. The response to phenylephrine returns fully within 48 hours in mouse aorta treated with PhB (147).

PhB has been suggested by some as the sole prophylactic strategy for the prevention of spasm in the RA. However, the levels of several other vasoconstrictors are raised following CABG and their contribution to the development of spasm should not be overlooked (91).

In addition to its α–adrenergic antagonistic properties, PhB also irreversibly antagonises the intracellular protein calmodulin (CaM) (148). CaM integrates the vasoconstrictor-mediated increases in intracellular calcium to activation of the myosin light chain kinase (MLCK), resulting in smooth muscle contraction (149). In previous work we found that PhB had no effect on the contractile response to ET 1, angiotensin II, vasopressin and the depolarisation induced by KCl (92). These results suggest that any interaction with CaM is unlikely to contribute to the antispasmodic action of PhB. Therefore PhB should not be used as the sole agent in the prevention of RA spasm. Taggart's group have since have also recommended this (150).

One of the concerns with the use of PhB is the possibility of an over expression of newly formed α adrenoceptors owing to up-regulation. This has been shown to occur in rat aorta smooth muscle cells that have been treated with PhB and then incubated in the presence of angiotensin II. This could be very important clinically as levels of

angiotensin II are raised postoperatively (151). The RA could become more sensitive to catecholamines, making spasm more likely.

Another problem with PhB is that there is an increased contractile response to angiotensin II in PhB treated arteries (146). The AT_1 and the α–adrenoceptor are both G-protein linked and may share components of intracellular calcium signalling (152). PhB pre-treatment may therefore contribute to vasospasm if it is mediated by non-catecholamine factors.

1.6.5 Other Treatments

Dobutamine is a β_1-receptor agonist, with β_2- and α_1-adrenoceptor agonist properties. It is frequently used following cardiac surgery because of its positively inotropic properties. Its vasodilator properties are due to an increase in cAMP in smooth muscle through activation of adenylyl cyclase and possibly the β–adrenergic pathway of relaxation such as activation of Ca^{2+}-sensitive potassium channels (136). Crawcoski *et al.* have showed that dobutamine is capable of relaxing IMA and GEA rings *in vitro* but was not as effective as sodium nitroprusside (133). Also at therapeutic levels it only caused between 0 and 25% relaxation (133). Dobutamine alone is therefore not a useful vasodilator clinically. However its action is potentiated by the phosphodiesterase inhibitors enoximone and milrinone (153) and a combination of drugs may be of some use clinically.

Potassium channel openers (KCO) are another drug of potential therapeutic value in the prevention of spasm in the RA. He and Yang have shown that the KCO aprikalim caused relaxation of the human IMA to KCl, U46619 and phenylephrine (154). Pre-treatment caused a decrease in the maximal response to phenylephrine but not to KCl or U46619, and shifted the concentration response curves of all three compounds to the right (154).

Various vasoactive substances are produced, released or altered following cardiac surgery. These substances include catecholamines, angiotensin II, thromboxane A_2, ET I, vasopressin, leukotrienes LTB4, LTC4, LTD4 and 5-HT (91). Many of these substances could cause spasm of the RA. Many inhibitors have been shown to prevent contraction of the RA to specific vasoactive substance (see table 1.5).

As it is not known which vasoconstrictor(s) are responsible for spasm in the postoperative period it is not possible to develop a strategy to prevent spasm by targeting a specific receptor. A more logical approach is to use drugs which block the pathways that lead to contraction of vascular smooth muscle or cause relaxation of it.

Vasoconstrictor	Inhibitor	Site of Action	Authors
Endothelin 1	BQ123	Endothelin A receptor	(84;112)
Angiotensin II	GR117289C	AT_1 receptor	(155;156)
Thromboxane A_2	GR32191B	TP receptor	(156)
Vasopressin	SR49059	V1 receptor	(155-157)

Table 1.5 – Inhibition of Contraction of the Radial Artery Due To Specific Vasoactive Substances

Batchelor *et al.* have shown in the IMA that the rho-kinase inhibitors HA1077 and Y27632 completely reverse sub maximal contractions of the thromboxane A_2 mimetic U46619 and ET 1 (158).

Ascorbic acid (vitamin C) is the main water soluble anti-oxidant in human plasma (159). It has been shown to reverse endothelial dysfunction in patients with ischaemic heart disease (160). This has been attributed to an enhanced synthesis or the prevention of the breakdown of nitric oxide (161). Drossos *et al.* have shown that vitamin C was a potent vasodilator of the RA *in vivo* and its effects were superior to diltiazem in patients with coronary artery disease (162). However Mangoush *et al.* found that ascorbic acid was only effective at preserving endothelium function after 72 hours incubation (163). This incubation period is not possible in the treatment of the RA prior to grafting.

Gene therapy offers a potential way of preventing spasm. This technique involves the use of an adenovirus to transduce the gene encoding for endothelial nitric oxide synthase. Cable *et al.* report RA rings undergoing transduction of this gene showed a significantly decreased contraction to KCl and to Prostaglandin $F_{2\alpha a}$ (56). This technique may also be used for the transfer of dominant negative constructs of Rho-A or Rho-kinase.

1.7 Areas of work to be investigated

1.7.1 Mechanisms of spasm

The cause of spasm is unknown, but is likely to be multi-factorial (158). Trauma following surgery, endothelial disruption, the presence of raised levels of vasoconstrictors following surgery (164) and temperature changes (90) are all possible causes. However spasm may occur many weeks or months following surgery when one would have expected any effects of surgery to have disappeared and levels of circulating vasoconstrictors to have returned to normal. Also, these raised levels of circulating vasoconstrictors are not high enough to cause contraction of RA rings *in vitro*. Elucidation of the possible mechanisms involved in spasm will aid in the design of successful strategies to prevent it.

Plasma NA levels are raised in the postoperative period from basal levels of 1.2-3.0 nM to levels of 6-15nM (165). Since sympathetic nerve endings release NA in close

proximity to the vascular smooth muscle of the arterial wall, interstitial concentrations are likely to be much higher than those measured in the plasma. Interstitial NA is difficult to quantify but the measured EC_{50} for NA mediated contraction in RA rings ranges from 0.04-0.7μM. This suggests that it is interstitial levels of NA rather than plasma concentrations that determine contractility.

It has been shown in saphenous vein grafts that after a week there is a decreased response to tyramine, an agonist that displaces NA from nerve endings, and a decrease in NA content (166). This suggests nerve endings have degenerated and therefore *in-vivo* would, during that degeneration, have the potential to degranulate and cause spasm. When vein grafts are placed under arterial pressure, although the contractile response at one week is decreased, the NA concentration response curve in vein grafts is shifted to the left. This decrease in contractile response is thought to be due changes in structure of the vessel wall. However, arterial grafts do not undergo the same structural changes that vein grafts do. The maximal contraction may therefore be preserved and if the same super-sensitisation (shift in the concentration response curve to the left) occurs, the RA would be particularly prone to spasm if nerve endings degenerate releasing NA.

The possibility that decaying nerve endings in the vessel wall may have the potential to cause raised local levels of vasoconstrictors and hence spasm of the RA will be investigated.

This will be investigated this by adding tyramine to human RA rings *in vitro*. Whether tyramine acts directly on Radial Artery Smooth Muscle Cells (RASMC), or via the α-

adrenoceptor will be looked at. Finally, possible ways of depleting nerve endings of NA prior to grafting using clinically-applied chemical treatments will be investigated.

1.7.2 Regeneration of New α-Adrenoceptors

PhB binds irreversibly to the α_1 adrenoceptor, the major adrenoceptor on the human RA (76). The vascular smooth muscle must regenerate new receptors *de novo*, before the response to NA is restored (167). The length of action of PhB persists for several days (144). However in smooth muscle cells cultured in the presence of angiotensin II, adrenoceptor regeneration occurs to levels beyond that originally present (168).

Angiotensin II levels are raised following cardiopulmonary bypass (165), therefore there may be an overshoot in adrenoceptor regeneration in the RA if it is pretreated with PhB prior to grafting. PhB would therefore prevent spasm in the initial postoperative period but then the RA would become more sensitive to catecholamines, and spasm occur later.

To investigate whether there is an overshoot in adrenoceptor regeneration; RASMCs will be cultured and treated with PhB. Concentration response curves will be obtained from cultured cells over a period of five days to measure the regeneration of functional adrenoceptors. These experiments will be repeated in the presence of physiological levels of a range of vasoconstrictors including angiotensin II.

If these initial studies show the anticipated overshoot, radioligand binding will be used to quantify the expression of α-adrenoceptors. Finally, organ culture techniques will be used to try and confirm the effects in the native artery.

1.7.3 Development of Alternative Treatments to PhB

PhB is a novel concept as it employs an irreversible treatment localised to the graft. However it deals only with the potential catecholamine component of spasm. Several other clinically approved drugs irreversibly target the coupling of receptor occupancy to contraction and therefore have a potentially broader effect against a variety of spasmogens. The effects of other clinically approved irreversible antagonists in the treatment of vasospasm will be studied by investigating their influence on contractility in arterial rings.

The actions of the inhibitors will be tested against vasopressin and angiotensin II. Levels of both of these vasoconconstrictors are raised in the post-operative period (165). Also normalised responses to both angiotensin II and vasopressin in the RA are stronger and more sensitive than in the IMA, and occur irrespective of the presence of endothelium (94;169;170), therefore it is important to be able to attenuate these responses. In addition, vasopressin-induced contraction in the RA is comparatively resistant to milrinone and GTN, two of the most commonly used vasodilator strategies (94). The inhibition of the vasopressin-induced contraction in RA grafts would also be particularly advantageous for surgeons, since they may wish to use vasopressin to treat hypotension in the postoperative period (104;171).

The two clinically approved irreversible antagonists that will be investigated are fluphenazine mustard and minoxidil sulphate. Fluphenazine is used clinically as an antipsychotic drug for the treatment of schizophrenia and other psyschoses. Minoxidil sulphate is used clinically to treat severe hypertension resistant to other drugs and hair loss.

Calmodulin Antagonist

Fluphenazine mustard is a cell permeable, irreversible antagonist of CaM (172). Its mechanism of action is by directly binding to CaM. Many intracellular Ca^{2+} signals are mediated by the ubiquitous calcium binding protein, CaM. Its function is to regulate a number of pathways. These include cyclic nucleotide metabolism, ion transport, protein phosphorylation/ dephosphorylation cascade, cytoskeleton function, cell proliferation and G protein mediated signalling (173). Binding of Ca^{2+} to CaM leads to the activation of several Ca^{2+}-dependent enzymes including MLCK, CaM-dependent protein kinase and calcineurin (173). Phosphorylation of myosin light chain by MLCK leads to interaction between actin and phosphorylated myosin light chain, activation of myosin ATPase, and subsequently to smooth muscle contraction. Inhibition of the activation of MLCK will prevent vasoconstriction

Potassium Channel Opener

Minoxidil sulphate binds irreversibly to the vascular ATP-sensitive K^+ channel (K_{ATP} channel) (174). K_{ATP} exist in a wide range of cells including endocrine cells, smooth

muscle cells, cardiac muscle cells and skeletal muscle cells (175). KCO relax smooth muscle by selectively increasing the membrane permeability to K^+. This repolarises or hyperpolarises the membrane decreasing the opening probability of voltage gated L- and T- type Ca^{2+} channels and inhibiting action potential formation. In addition KCO may accelerate the clearance of intracellular free Ca^{2+} via the Na^+/Ca^{2+} exchange (176).

Potent vasodilatation with nicorandil, a combined KCO and NO donor, has been reported against endothelin-mediated contraction in the RA (177). Pinacidil, which shares a common binding site with minoxidil sulphate and nicorandil on the K_{ATP} channel, has also demonstrated its ability to reverse phenylephrine-induced contraction in human RA (178). Therefore the activation of K_{ATP} channels would be expected to reverse membrane depolarisation, inhibit calcium influx across the plasma membrane and relax the pre-contracted RA.

Rho-kinase Inhibitor

Finally the rho-kinase pathway (see figure 1.4) will be investigated as another common pathway that could potentially be blocked to prevent spasm. The sustained phase of vascular smooth muscle contraction is thought to involve Ca^{2+} sensitization (179). It is believed to be this phase that initiates clinical vasospasm in cerebral arteries (180). The major mechanism of the Ca^{2+} sensitization of contraction is through the inhibition of the smooth muscle myosin light chain phosphatase, resulting in increased myosin light chain phosphorylation and smooth muscle contraction at a constant intracellular calcium level (179). It has been recognised that the monomeric G protein Rho and its downstream

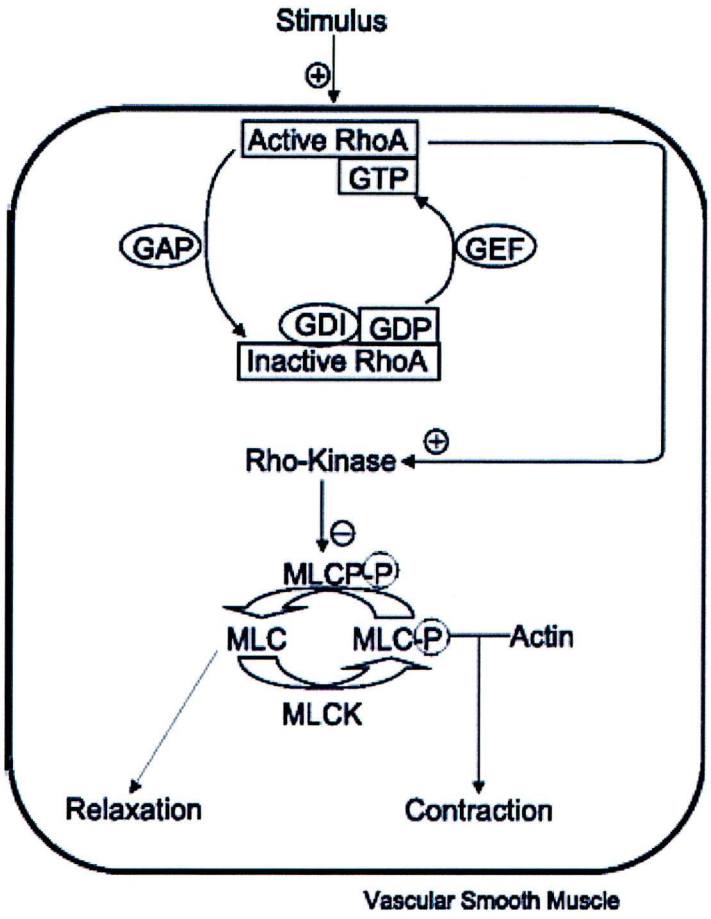

Figure 1.4 - Simplified diagram showing regulation of rhoA/rho-kinase activity. Stimulation of the vascular smooth muscle cell activates rhoA by guanine-nucleotide exchange factors (GEFs), which facilitate exchange of GDP for GTP, thus rendering rhoA active; GTP-bound rhoA activates rho-kinase. This phosphorylates the myosin-binding subunit of MLCP, thus inactivating it. Myosin light chain (MLC) phosphorylation is regulated by MLC kinase (MLCK, which phosphorylates and activates MLC) and MLC phosphatase (MLCP, which dephosphorylates and inhibits MLC). MLC phosphorylation leads to increased contractile tone via enhanced cross-bridging with actin.

target Rho-kinase can participate in sustained vasoconstriction by phosphorylating and inhibiting myosin binding (181). Rho-kinase has been proposed to play a variety of vascular smooth muscle disorders including hypertension, coronary and cerebral vasospasm (180;182;183).

In summary, the initial aim of this thesis is to investigate whether decaying nerve endings in the vessel wall may have the potential to cause raised local levels of vasoconstrictors and hence spasm of the RA. Next the safety of the currently used irreversible α-adrenergic antagonist, PhB, will be studied. This will be examined with respect to whether there is an overshoot in α-adrenoceptor responses following PhB treatment. Finally, the efficacy of an irreversible KCO, an irreversible CaM antagonist and a rho-kinase inhibitor will be investigated.

Chapter 2

Methods and Materials

Ethical committee approval for the project was obtained from the Liverpool Research Ethics Committee (ref 02/06/094/A) and the Cardiothoracic Centre, Liverpool NHS Trust Research and Development Committee. Samples of RA, surplus to requirement for CABG, were collected at the Cardiothoracic Centre, Liverpool, with fully informed patient consent.

2.1 Radial Artery Harvest Techniques

The RA was harvested with surrounding fat and the two satellite veins using diathermy and titanium surgiclips to achieve haemostasis on the RA in 74 patients, and surgiclips only in 15 patients (see figure 2.1). Depending on the practice of the surgeons concerned, radial arteries were treated in the theatre with either 1.6 mM papaverine (Martindale Pharmaceuticals, Romford, UK) or 6 mM PhB (Goldshield Pharmaceuticals Ltd., Croydon, UK) in a solution of the patients' whole blood containing 100 i.u. heparin/ml for 30 minutes. Arterial sections surplus to surgical requirements were collected from theatre into Dulbecco's Modified Eagle Media (DMEM) (Invitrogen,UK) on ice and immediately transported to the research laboratories.

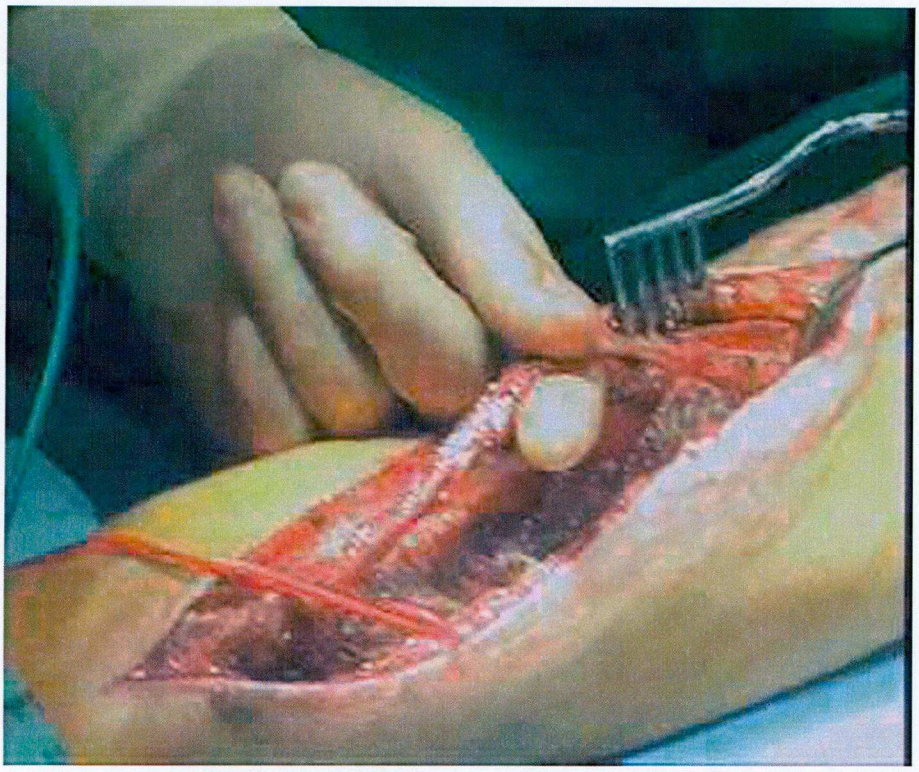

Figure 2.1 – Photograph showing harvesting of the RA with surrounding fat and satellite veins (patients consent obtained)

2.2 Patient Details

RA samples were collected from 89 patients. The clinical characteristics of these patients are given in table 2.1. In 40 patients, distal segments of RA were collected and in 49, proximal segments. 44 radial arteries were pre-treated with 6 mM PhB and 45 with 1.6 mM papaverine.

Total number of Patients	89
Mean age (years)	64
Sex ratio (Male: Female)	65:14
Risk Factors (n)	
Current Smokers	13
Ex-smokers	49
Non-smokers	27
Hypertension	50
Diabetes mellitus	23
Peripheral Vascular disease	12
Previous CVA or TIA	7
Preoperative Medication (n)	
β blockers	70
Calcium channel blockers	44
ACE inhibitors	31
Nitrates	49
Potassium channel openers	28

Table 2.1 - Patient Characteristics

2.3 Materials

All tissue culture reagents were purchased from Invitrogen Ltd (Paisley, UK) and plastic ware was obtained from Sarstedt (Leicester UK). Reagents were purchased from DBL (Warwick UK), Martindale Pharmaceuticals (Romford, UK), Goldshield Pharmaceuticals Ltd (Croyton, UK), Abbott Pharmaceuticals Ltd (Maidenhead, UK), Sigma Chemicals (Poole, UK) , Merck Ltd (Nottingham, UK), Molecular Probes (Oregan, USA), Novacastra Laboratories (Newcastle-Upon-Tyne, UK) and Calbiochem (Nottingham,UK) (See table 2.2).

Chemical	Company
Angiotensin II	Sigma Chemicals
arg-vasopressin	Sigma Chemicals
Bovine Serum Albumin	Sigma Chemicals
CaCl2	Sigma Chemicals
D-glucose	Sigma Chemicals
DMEM	Invitrogen
Endothelin I	Sigma Chemicals
fluo 4	Molecular probes
Foetal bovine Serum	Sigma Chemicals
Glucose	Sigma Chemicals
Goat anti-mouse IgG FITC labelled secondary antibodies	Sigma Chemicals
Glyceryl trinitrate	DBL
KCl	Sigma Chemicals
L-Glutamine	Sigma Chemicals
$MgSO_4$	Sigma Chemicals
Minoxidil sulphate	Merck
Mouse anti-human α smooth muscle actin antibodies	Novacastra Laboratories
Na_2HPO_4	Sigma Chemicals
NaCl	Sigma Chemicals
$NaHCO_3$	Sigma Chemicals
Noradrenaline	Abbott Pharmaceuticals Ltd
Papaverine	Martindale Pharmaceuticals
Phenoxybenzamine	Goldshield Pharmaceuticals Ltd
Pluronic F-127	Sigma Chemicals
Polyclonal rabbit IgG against S100 protein	Dako
Reserpine	Merck
Sodium Bicarbonate	Sigma Chemicals
Sulphinpyrazone	Sigma Chemicals
Tyramine	Sigma Chemicals
Y27632	Merck

Table 2.2 - Reagents Used

2.4 Culture of Human Radial Artery Smooth Muscle Cells

RASMC were grown from explants using a method based on that described by Kirschenlohr *et al.* (184). Small sections of RA (0.5-3.0cm), surplus to CABG, were obtained with informed patient consent. They were then transferred to the tissue culture laboratory in ice cold DMEM containing antibiotic (100U/ml penicillin, 100μg/ml streptomycin sulphate and 0.25μg/ml amphotericin B). The artery was opened longitudinally and the endothelium scraped off using a sterile scalpel. If visible, the intima was lifted off using a pair of watchmakers' forceps. The tunica-media containing the smooth muscle was then removed and gently chopped, using scissors, into pieces of 1-2mm. These pieces were re-suspended in culture medium (DMEM containing 4mM L-glutamine, 2.5mM glucose and 4.5mM sodium bicarbonate) supplemented with 20% foetal bovine serum (FBS) and antibiotic (100U/ml penicillin, 100μg/ml streptomycin sulphate and 0.25μg/ml amphotericin B), and transferred to $25cm^3$ tissue culture flasks. The pieces were spread evenly over the bottom of the flask and the medium aspirated leaving only a thin film, sufficient to keep the tissue moist. The flask was left in a humidified tissue culture incubator at 37°C for a minimum of 2 hours to allow the pieces to adhere. After this time 1.2mls of culture medium was added. After 3 days any unattached tissue was removed and the media changed. Thereafter media was changed 3 times a week. When confluent, explant pieces were gently floated off in an excess of media and the cells allowed to recover for 2 days before subculturing. RASMC were detached using trypsin/EDTA solution and reseeded into a $75cm^3$ flask. When confluent

the RASMC were then further passaged into three further $75cm^3$ flasks or onto a 96 well plate for imaging. Cells were routinely used within the first four passages.

2.5. Antibody Staining of Human Radial Artery Smooth Muscle Cells

Smooth Muscle cells were cultured to confluency on glass cover slips and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS). Fixed cells were then treated with 50mM NH_4Cl for 10 minutes and then permeabilised with 0.1% Triton X-100 for 10 minutes. Cover slips were then incubated with mouse anti-human α smooth muscle actin antibodies (Novacastra Laboratories, Newcastle-Upon-Tyne, UK) at 1:50 dilution overnight in the presence of 1% bovine serum albumin (BSA) and then incubated with goat anti-mouse IgG (Fab specific) flourescein isothiocyanate (FITC) labelled secondary antibodies (Sigma, Poole, UK), at 1:500 dilution overnight in the presence of 1% BSA. Following washing cover slips were mounted on microscope slides using Gelvatol Airvol mountant and positive staining was visualised using a x60 objective of LEITZ Diaplan fluorescence microscope (Leitz-Wetzlar, Wetzlar, Germany) housed in the Department of Anatomy and Cell Biology, University of Liverpool, using standard FITC filters. Negative controls where control mouse serum was used in place of the primary antibody were included to confirm positive staining.

2.6 Calcium Imaging

Radial artery smooth muscle cells were cultured to confluence on five 96 well plates (Sarstedt, Inc. Leicester UK). All plates were fed 3 times a week and always on the day prior to imaging. All calcium imaging was performed in HEPES-Buffered Saline (HBS) composed of: HEPES, 20mM; NaCl, 145mM; D-glucose, 10mM; $CaCl_2$, 1mM ; pH 7.4 at 37°C.

Initially the cells were placed on serum free culture media for at least 48 hours prior to commencing the experiment to maintain a quiescent phenotype. However the cells had a tendency to lift on serum free media even in the presence of 0.1% BSA. Results obtained from cells placed on serum free media for 48 hours, and cells not placed on serum free media were identical for the initial experiment where α adrenoceptor regeneration was investigated not in the presence of any vasoconstrictor, therefore all further experiments were performed without placing the cells on serum free media for 48 hours prior to the commencement of the experiment.

Vasoconstrictor-induced changes in intracellular calcium concentration were recorded in RASMC loaded with the calcium sensitive fluorescent dye, fluo 4. Cells were loaded with 2μM fluo 4 (Molecular Probes, OR, USA), 0.025% of the detergent Pluronic F-127 at 37°C for 120 min, in 100μl loading buffer consisting of HBS plus sulphinpyrazone, 200μM, to prevent dye leakage, and 1 % BSA. After loading, extracellular dye was aspirated and the cells maintained in 50μl dye-free HBS. Agonist stimulated changes in

intracellular calcium were monitored using a Wallac 'Victor' 1420 Multilabel counter (Perkin-Elmer, UK). A single row (12 wells) was imaged at a time. Cells were then excited at 490nm and the resulting fluorescence monitored at 520nm. Then 50μl of agonist was added to each well and the fluorescence measurement repeated. The change in fluorescence was calculated by subtracting the mean of the first reading from the mean of the second reading. Once this was complete the dispenser was flushed with the next agonist and the process repeated for the next row of the 96 well plate.

It was noted that during the course of imaging a plate there was a gradual increase in background fluorescence. The explanation for this was thought to be dye leakage. To confirm this, the experiment was repeated with 1mM manganese chloride being added to the well, to quench any extracellular dye, just prior to the initial fluorescence reading on each row.

The experiments were also repeated, but instead of the addition of an agonist to each row, buffer was added to each row to assess the effect of dye leakage on the change in fluorescence. All other aspects of the experiment were kept the same including flushing of the dispenser to keep the time period of the experiment the same. This allowed a value to be calculated for the contribution of dye leakage to change in fluorescence of each row with time.

2.7. Organ Bath Techniques

Organ bath contraction studies were carried out at 37°C in oxygenated Krebs solution composed of; NaCl, 118 mM; KCl, 4.7 mM; Na_2HPO_4, 1.2 mM; $MgSO_4$ 1.2mM; D-glucose, 10 mM; $CaCl_2$, 1mM; $NaHCO_3$ 25mM.

2.7.1 Pre-tensioning of Rings

Various methods of pre-tensioning vascular rings have been described. These methods are based around variations of two separate methods initially described by Mulvany and Halpern (185) and Cohen (186).

We adopted a method, based on that described by Mulvany, similar to that used by He and Yang (170;176;187-193) and that we had used in previous studies (92). The length of time rings were stretched and relaxed prior to use was decreased from 30 minutes to 20 minutes in order to aid in the processing of more than one sample per day. This decrease in time appeared to have no effect on the maximal tension pulled by the ring. Maximal tensions pulled after 20 minutes and 30 minutes were 9.87g±0.43 vs. 9.12g±0.44 respectively (p=0.25) (See figure 2.2).

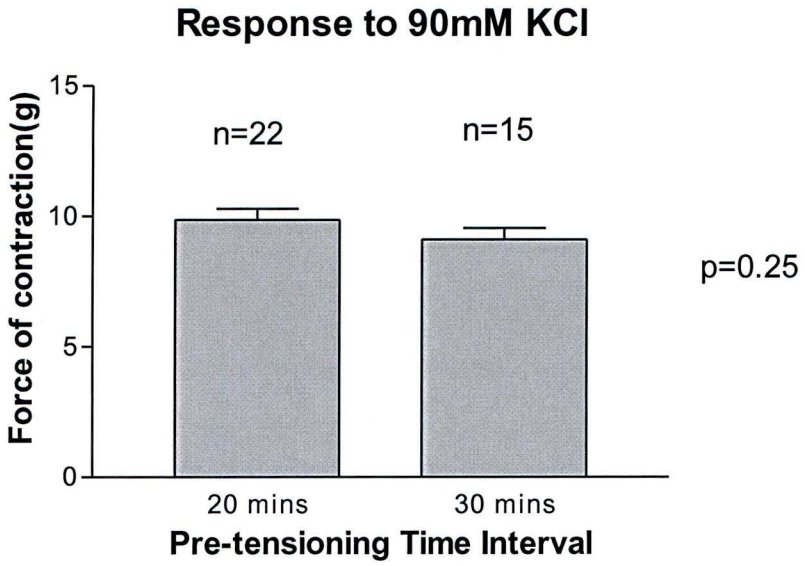

Figure 2.2 – Bar chart showing maximal tension pulled in response to 90mM KCl by proximal RA rings, pre-tensioned for 20 minutes and 30 minutes respectively. Bars represent mean ±SEM.

Samples of artery were trimmed of connective tissue and cut into 2-3mm rings using a scalpel. Prior to mounting in the organ bath, a ring was selected at random and divided along its length. The length of this strip of artery was then measured with a micrometer to give the relaxed internal circumference. The remaining rings were mounted in 25mls Krebs solution between two fine wire stirrups connected to a force transducer and changes in isometric force were recorded on a PowerLab 16SP data recording system connected to an Octal ML119 bridge amplifier (AD Instruments Ltd, Chalgrove, Oxfordshire, UK) (See figure 2.3). Each ring was stretched to a tension equivalent to 100mmHg and the tension allowed to decline for 20 minutes, after which it was

readjusted again to a similar tension and allowed to decline for a further 20 minutes. Following this the tension was relaxed to approximately 90% of their maximal circumference for a further 20min prior to the addition of agonists.

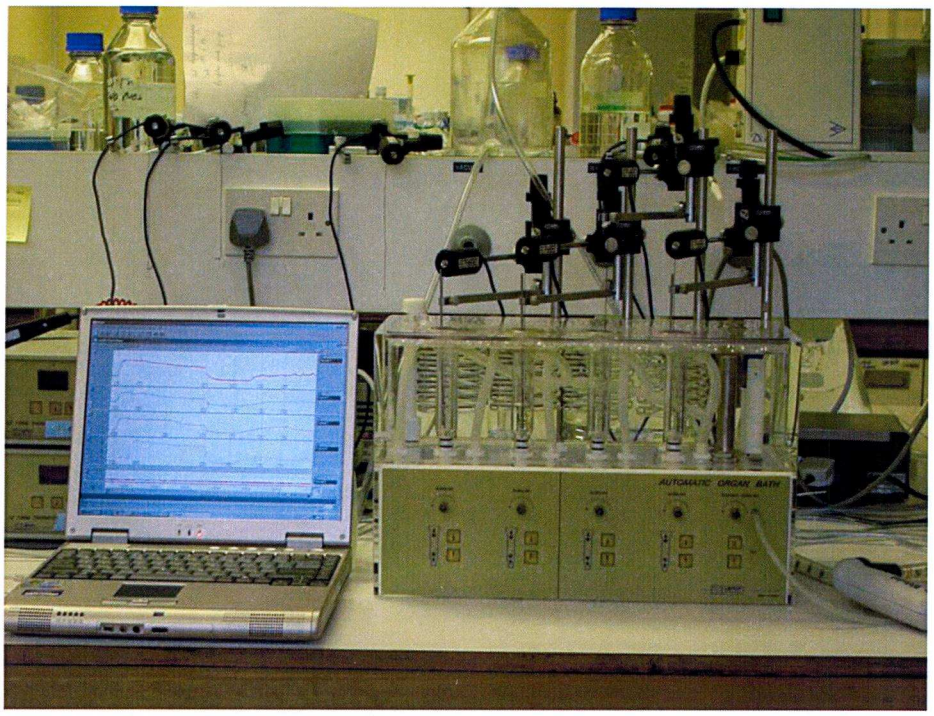

Figure 2.3 – Organ bath and recording system

The wall pressure of the RA ring was calculated from the following equations.

Wall Tension per unit area – T

Vessel length – g

Force exerted by vessel on force transducer – F

Transmural pressure – P

Internal Circumference – L , L_R – relaxed , L_s – stretched

Stretch – S

$$T=F/2g$$

$$P=2\pi T/L$$

$$L_s=L_R+2s$$

We have made the following assumptions:

1. The ring is a thin walled tube.
2. T is unaffected by the curvature caused by suspension of the ring in the organ bath.

Details of individual organ bath experiment are given in the relevant chapter.

2.8. Regeneration of Radial Arteries in Organ Culture

Distal sections of RA pre-treated for 30 minutes with 6mM PhB were collected from theatre. This has been shown to abolish the NA-mediated contraction (193). The RA was cut into 6 rings 2-3mm in length. One of the rings was mounted in the organ bath as described below and responses to 90mM KCl, increasing concentrations of NA (0.1μM, 1μM, 2μM, 4μM, 10 μM and 100 μM), and 100nM vasopressin recorded. The other 5 rings were placed under 6g of tension, provided by an external weight, in a petri dish with DMEM (with 4mM L-glutamine, 2.5mM glucose and 4.5mM sodium bicarbonate) containing 20% FBS and antibiotic. The petri dish was then placed in the tissue culture incubator. At 24 hour intervals a ring was removed and the organ bath experiment repeated. The media was changed every 24 hours.

2.9 Statistical Analysis

Analyses were carried out using the programs Arcus QuickStat Biomedical (Hearne Scientific Software, Dublin, Eire).or Graphpad Prism version 3.02 (Graphpad Software, California, USA).

In chapters 3 and 4 data is expressed as mean plus or minus standard error of the mean (SEM). In chapter 5 data is presented as mean ± standard deviation of the mean (SD), which was the preferred method by the journal in which this chapter was published. A p value of less than 0.05 was considered statistically significant. n refers to the number of independent samples tested

Comparison between two groups were made using a paired 2 tailed student's t test A one way analysis of variance (ANOVA) and a Bonferroni correction was used for multiple group comparisons.

Concentration response curve were constructed and EC_{50} calculated using non-linear regression methods on Graphpad Prism. This was used to calculate EC_{50}.values. They are expressed as mean (with 95% confidence intervals). EC_{50}.values are compared using a one way ANOVA.

Chapter 3

Degenerating Nerve Endings in the Wall of the Human Radial Artery May cause Spasm

3.1 Introduction

When vein grafts are placed under arterial pressure, although the contractile response at one week is decreased, the NA concentration response curve in vein grafts is shifted to the left (194). This decrease in contractile response is thought to be due to changes in the structure of the vessel wall. However, arterial grafts do not undergo the same structural changes that vein grafts do. The maximal contraction may therefore be preserved, and if the same super-sensitisation occurs, the RA would be particularly prone to spasm if nerve endings degenerate releasing NA. Consequently sympathetic nerve endings may be a potential source of spasmogenic NA.

The aim of this work is to investigate the possibility that degenerating nerve endings in the vessel wall of the RA have the potential to cause contraction of the RA. If this is a possibility, then methods of preventing this will be investigated

The presence of sympathetic nerve endings in sections of human RA will be confirmed by staining for S100 protein. The response of RA rings to tyramine will then be tested in the organ bath. To confirm that any response is mediated by the α-adrenoceptor, PhB will be used to try and block the response. Tyramine will then be added to cultured RASMC to see whether it exerts a direct response on the cells or whether it is acting via another method. Finally, various chemical treatments will be investigated to determine ways nerve endings could be depleted of NA prior to grafting.

The drugs that we will use to try and deplete nerve endings are guanethidine monosulphate, reserpine and 6 hydroxy-dopamine. Guanethidine is a drug which is used clinically to treat a hypertensive crisis (195). Guanethidine is taken up into the noradrenergic nerve terminal by the uptake 1 system. Once inside it blocks the release of NA in response to an action potential.

Reserpine is a centrally acting anti-hypertensive. It is available in the USA but no longer used clinically in the UK. It is an alkaloid that comes from the shrub, Rauwolfia. It acts by blocking the transport of NA into the synaptic vesicles. The NA accumulates in the cytoplasm where it is broken down by monoamine oxidases (MAO). The NA content of the tissue drops and synaptic transmission is blocked.

6 hydroxy-dopamine is similar in structure to dopamine except that it possess an extra hydroxyl group. It is taken actively up by noradrenergic nerve terminals, where it is converted to a reactive quinine, which destroys the nerve terminal. It is available in this country for experimental use only.

.

3.2 Methods and Materials

Samples of RA were obtained from patients undergoing CABG. They were harvested as described in chapter 2. 49 rings from 27 patients were used in total.

3.2.1 S100 stain

6 RA rings from 3 patients were used. RA segments were embedded in paraffin wax. From each block 5μm sections were cut. Sections were washed overnight with 0.01M phosphate-buffered saline containing 0.5% Triton X-100. After washing with PBS, the specimens were treated with the same buffer that contained 0.3 % H_2O_2, and washed with PBS again. They were incubated for 1 hour with non-immune 2% goat serum. The specimens were then incubated with polyclonal rabbit IgG against S-100 protein (Dako, Ely UK) at 1:1000 dilution, overnight at 4^0C. After the incubation, samples were treated with biotinylated antibody against rabbit IgG raised in 0.5% goat serum (Vector, Peterborough, UK), for 1 hour at room temperature. Immunoreactive sites were visualised by incubation with Tris-HCl buffer that contained 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2. Immunostained specimens were mounted on slides coated with chrome alum and gelatin, air-dried, dehydrated in an ethanol series, cleared with xylene, and sealed under coverslips.

For immunohistochemical controls, normal rabbit IgG was used instead of polyclonal rabbit IgGs against S-100 protein. Additional sections were stained with haematoxylin and eosin.

3.2.2 Organ Bath Experiments

Proximal RA samples were used for these organ bath experiments. They had been pre-treated in theatre with 1.6mM papaverine. RA rings were set up and pre-tensioned in the organ bath as described in chapter 2. All rings were initially contracted with 90mM KCl to test for functional contractility. Responses were terminated by washing with three complete changes of media. 7 rings from 3 patients were stimulated with increasing concentrations of tyramine to construct a concentration response curve.

The effect of PhB on tyramine induced contraction was investigated using 14 rings from 5 patients. In order to block any α adrenergic response half of the rings were treated with 0.1mM PhB for 30 minutes and half left untreated. Following washout, tyramine 500μM was then added to all the rings and the strength of contraction noted. Any contraction was then reversed by increasing concentrations of GTN.

To investigate tyramine-induced tachyphylaxis, 500μM tyramine was then added to all the rings and the strength of contraction noted. The response was washed out and the tension allowed to return to basal levels. Rings were then stimulated with 100μM NA to check that any failure to respond to the second addition of 500μM tyramine was due to depletion of NA in the nerve endings and not receptor desensitisation.

Finally, 100nM vasopressin was added to the rings to confirm they still contracted.

To determine whether RA rings had the potential to replenish NA following depletion with tyramine, rings were initially treated with tyramine, washed out and then 100uM NA was added. Following this a further dose of 500uM tyramine was added to see if the rings responded to this second dose of tyramine following exposure to high concentrations of NA.

To investigate the effects of guanethidine, reserpine and 6 hydroxy-dopamine on the tyramine response, a further set of experiments were carried out using 14 rings (1 discarded) from 3 patients. The rings were either pre-treated with guanethidine, reserpine or 6 hydroxy-dopamine for 15 minutes and the response to 500μM tyramine recorded

3.2.3 Calcium Imaging

RASMC were cultured to confluency on 96 well plates as described in chapter 2. Cells were washed with HBS and the cells loaded with the calcium sensitive dye Fluo 4 in a total volume of 100μl of loading media per well which contained 2μM Fluo 4, 200μM sulphinpyrizone (SPZN), 0.025% F127, 1% BSA. Cells were loaded for 2 hours at 37°C. Following this, the loading media was aspirated and 50μl of buffer placed in each well. Cells were excited at 490nm and changes in fluorescence monitored at 525nm using a Wallac 'Victor' 1420 Multilabel counter (Perkin-Elmer, UK)
The background fluorescence was measured in the first row of 12 wells (5 readings per well). Following this 50μl of buffer was added to each of these wells and the peak

change in fluorescence measured (5 readings per well). This was repeated for rows 2 to 4 with the addition of 50µl of 1mM tyramine, 200µM NA or 200nM angiotensin II respectively.

3.3 Results

3.3.1 S100 antibody staining

Immunohistochemistry on 6 rings from 3 patients with S100 antibody confirmed the presence of nerve endings in all sections (see figure 3.1).

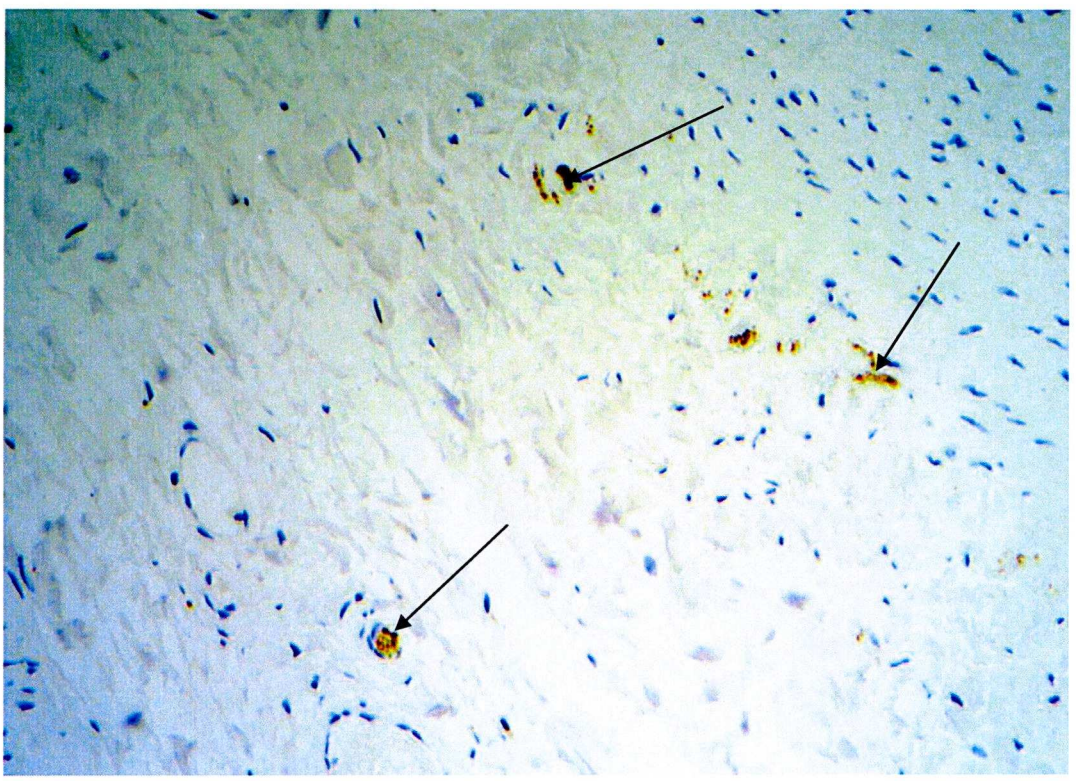

Figure 3.1 – Medium power microscopy showing nerve endings in RA sections stained with polyclonal rabbit IgG against S-100 protein (⟶)

3.3.2 Organ Bath Experiments

Patient characteristics are shown in table 3.1.

Total number of Patients	11
Mean age (years)	61
Sex ratio (Male:Female)	9:2
Risk Factors (n)	
Current Smokers	3
Ex-smokers	6
Non-smokers	2
Hypertension	7
Diabetes mellitus	3
Peripheral Vascular disease	1
Previous CVA or TIA	0
Preoperative Medication (n)	
β blockers	8
Calcium channel blockers	5
ACE inhibitors	4
Nitrates	8
Potassium channel openers	2

Table 3.1 – Patient characteristics

A concentration response curve for tyramine was constructed (see figure 3.2). The EC_{50} was 272±33μM. A concentration of 500μM tyramine was therefore used for the rest of the experiments in this chapter to achieve a maximal contraction.

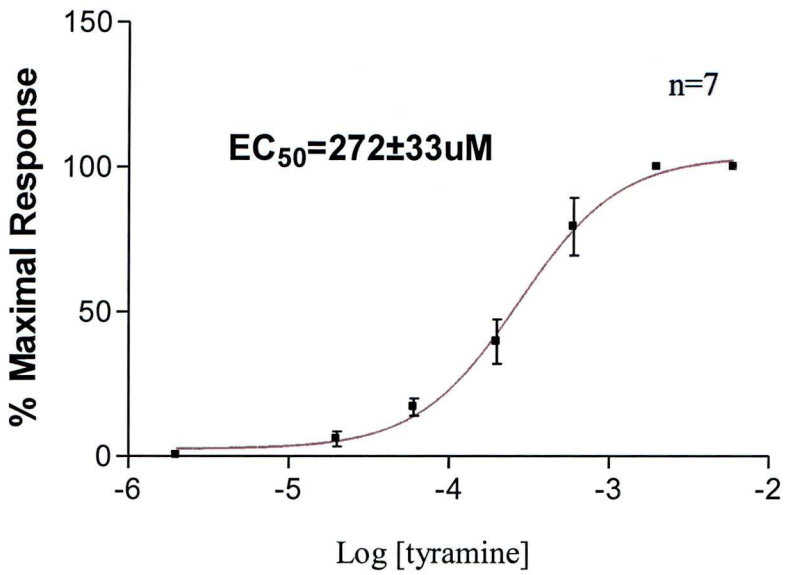

Figure 3.2 - Tyramine concentration response curve. Increasing concentrations of tyramine were added to 7 rings from 3 patients. Responses are shown as a percentage of maximal response to tyramine. Mean values for each dose are plotted. Error bars represent SEM.

Using 14 rings from 5 patients the effects of PhB on tyramine-induced contraction were compared. Half of the rings were treated with 0.1mM PhB and half left untreated. All

rings responded to 90mM KCl. The mean force of contraction was 3.97±0.40g in the untreated group and 4.10±0.57g in the PhB treated group (figure 3.3). There was no significant difference between the PhB and untreated rings. (p=0.76).

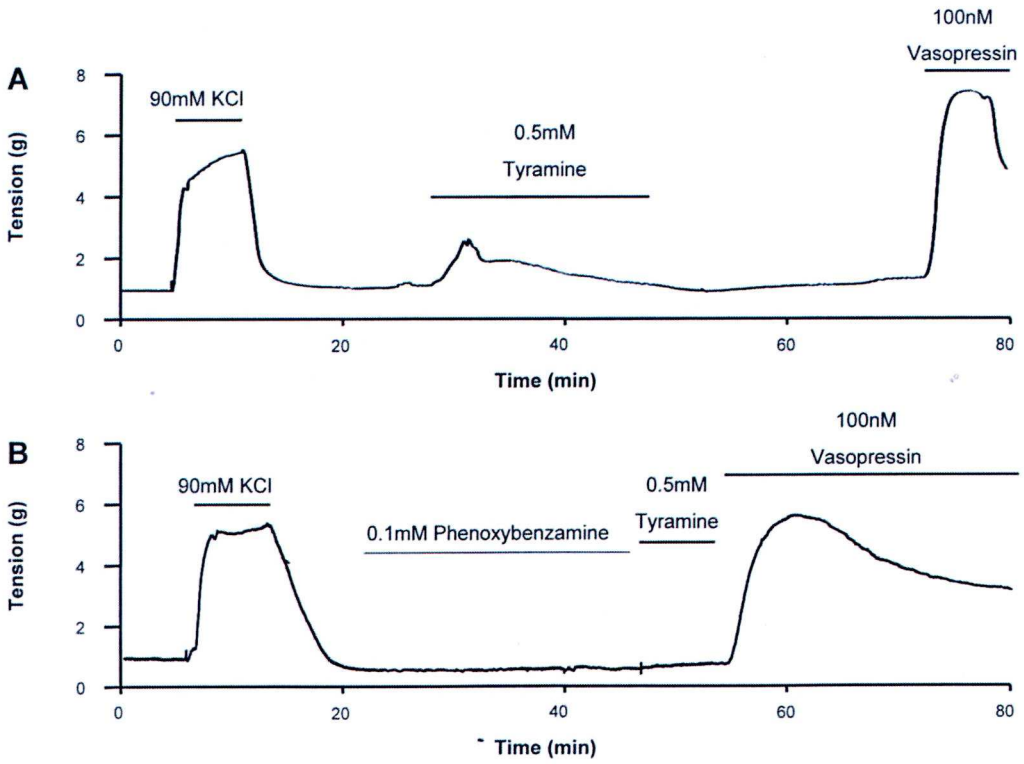

Figure 3.3 – Response of PhB treated and untreated rings to tyramine. Representative organ bath traces from individual rings of human RA showing an initial response to 90mM KCl. 0.1mM PhB was added to ring B for 30 minutes then washed out. 500μM tyramine was added to both rings. Following washout 100nM vasopressin was added to the rings.

All the rings in the untreated group showed a significant increase in tension in the presence of 500μM tyramine 2.76±0.40g, 64.5±8.7% of the 90mM KCl response (figure 3.4). This did not occur in the presence of PhB ($p<0.0005$). To confirm functional contractility at the end of the experiment, all rings responded to 100nM vasopressin at the end of the experiment. There was no statistical difference between the untreated and PhB treated rings 4.31±0.87g; 102.14±12.87 %KCl vs. 3.94±0.70g; 92.80±8.57 %KCl response ($p=0.51$) (Figure 3.4).

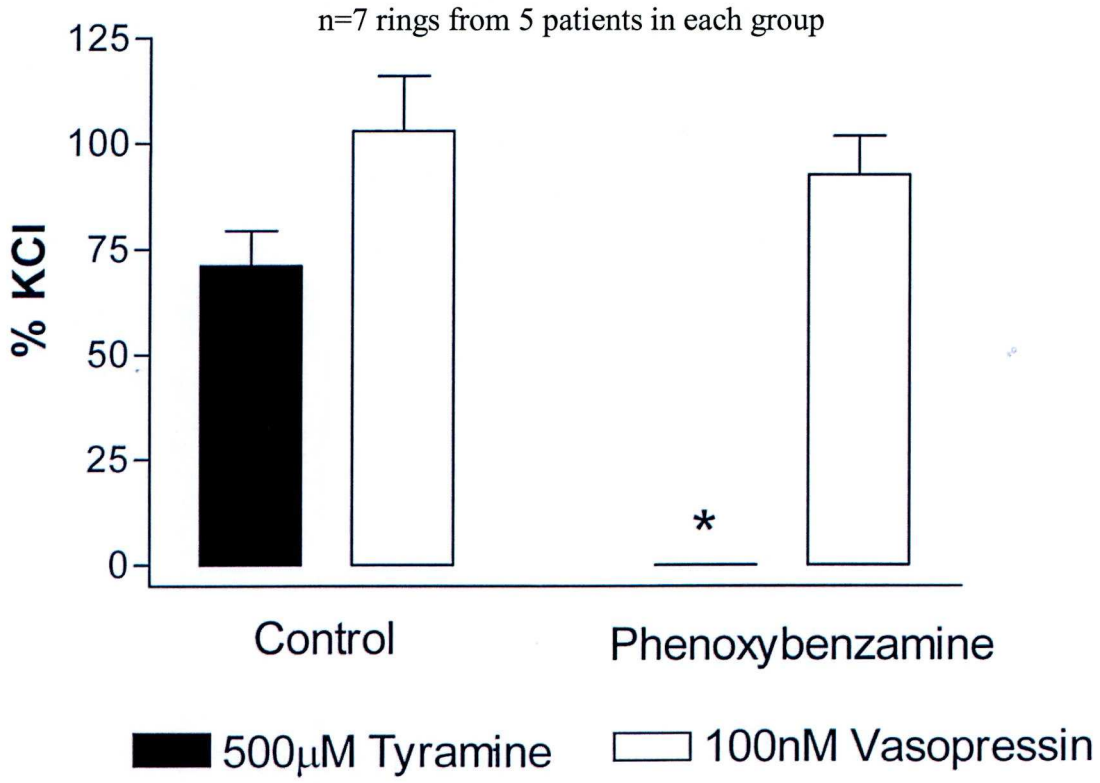

Figure 3.4 –Comparison of Tyramine and Vasopressin responses in PhB treated and untreated rings. Responses are expressed as a percentage of the initial 90mM KCl response. Bars represent mean ±SEM. * denotes significant difference versus control ($p<0.0005$)

The response was reversed by increasing concentrations of GTN (Figure 3.5) however relatively high concentrations were necessary to completely reverse the contraction.

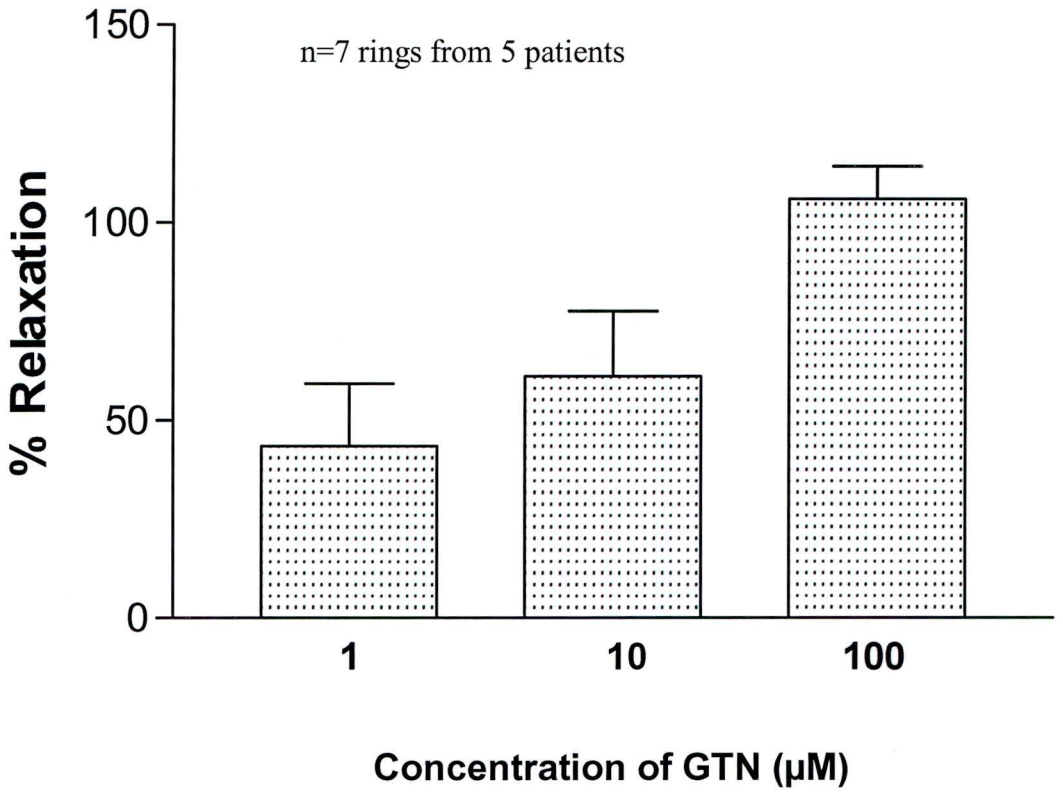

Figure 3.5 – Relaxation of tyramine induced contractions in the human RA by increasing concentrations of GTN. Values are expressed as a percentage of the tyramine induced contraction. Bars represent mean ±SEM.

To test for tyramine-induced tachyphylaxis, following a washout, a further 500μM of tyramine was then added to all the rings and the strength of contraction noted. This did not cause an increase in tension in any of the rings tested. However, after this all rings responded to 100μM NA following washout giving a response, not significantly different from the response to tyramine 71.42±5.2 %KCl vs. 85.7±7.01 %KCl (figure 3.6 and 3.7).

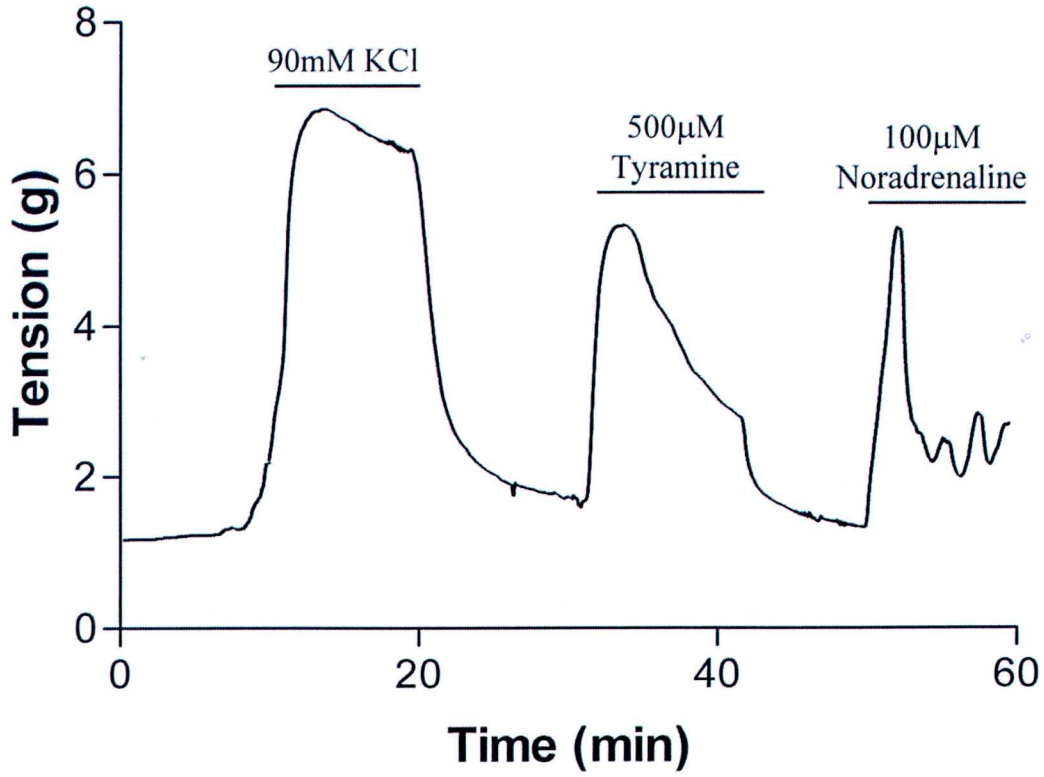

Figure 3.6 – Representitive organ bath trace showing comparison of responses to 90mM KCl, 500μM tyramine and 100μM noradrenaline in a human radial artery ring. Each response was reversed by 3 complete changes of media.

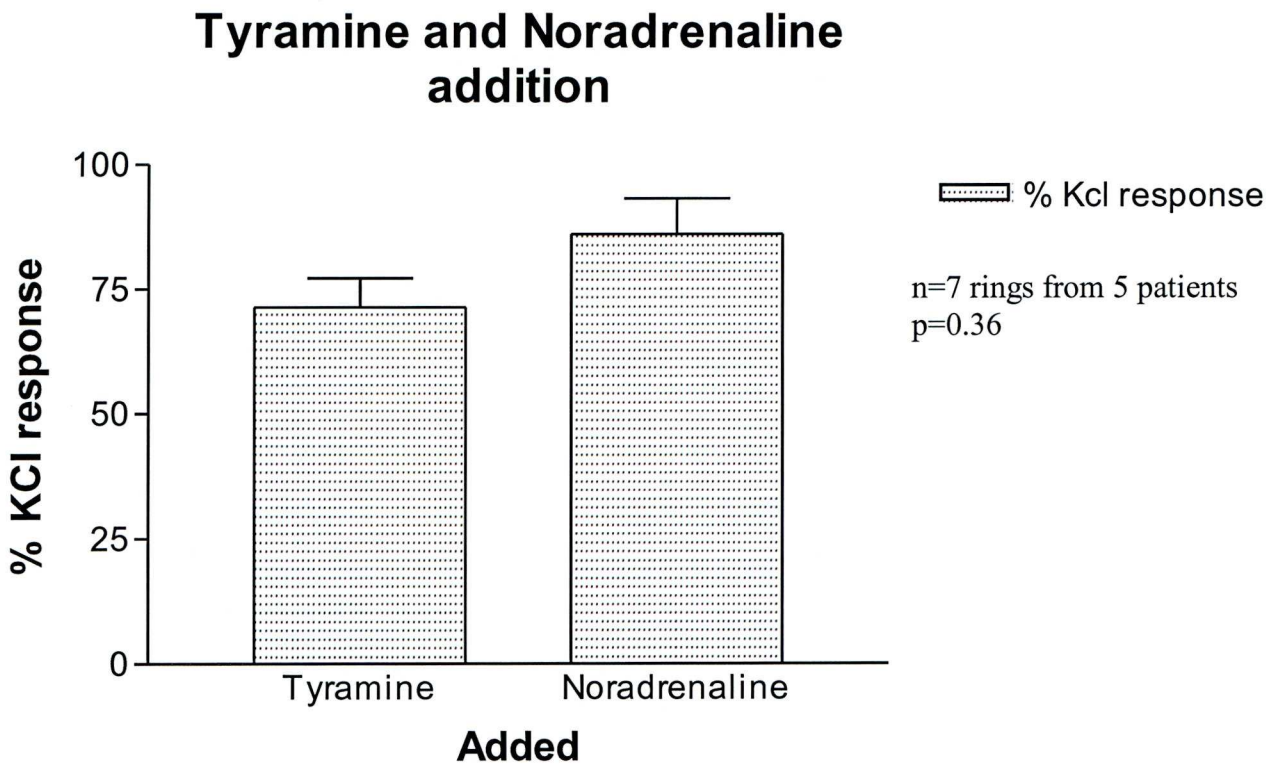

Figure 3.7 – Comparison of maximal 500 μM tyramine and 100μM noradrenaline responses. Responses are expressed as a percentage of the maximal 90mM KCl response. Bars represent mean ±SEM.

In experiments designed to deplete nerve endings of NA by chemical treatment, sections of RA from each patient was pre-treated with either 5μM reserpine, 1mM guanethidine or 2mM 6-OH dopamine for 15 minutes prior to stimulation with tyramine. These drugs were washed off by 3 complete changes of media prior to the addition of tyramine. The responses are shown in figure 3.8. Reserpine significantly decreased the contractile response to tyramine ($p<0.05$).

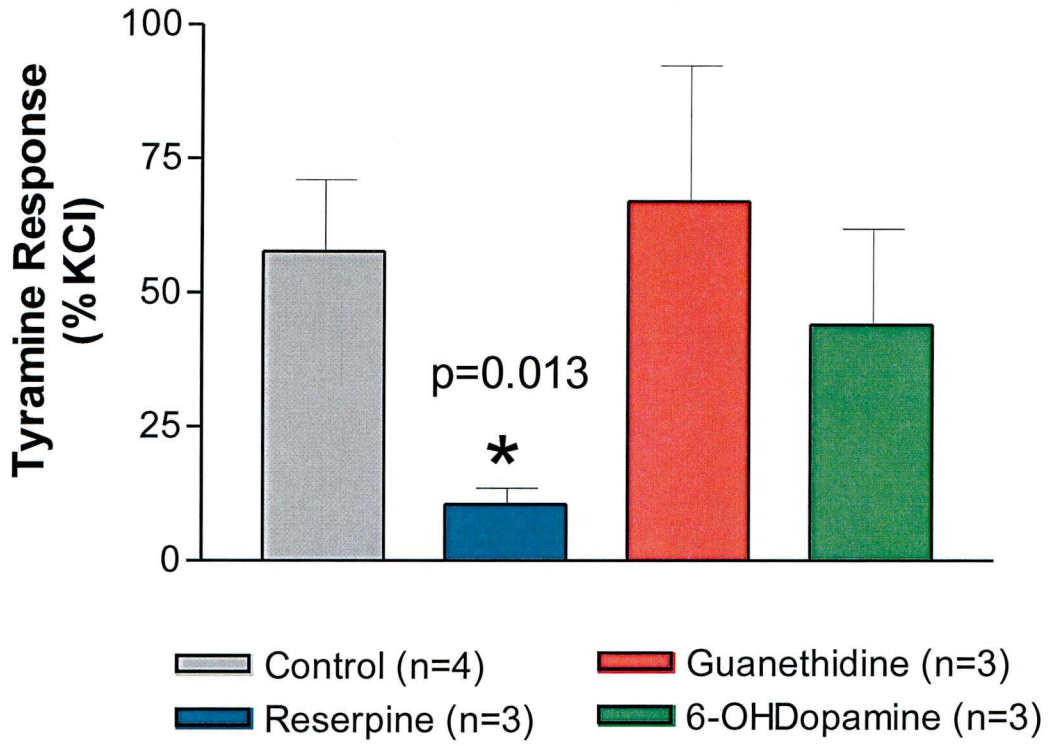

Figure 3.8 – Tyramine responses in human radial artery rings; in controls and in rings treated for 15 minutes with either 5μM reserpine, 1mM guanethidine or 2mM 6-OH dopamine. Responses are expressed as a percentage of the initial 90mM KCl response. Bars represent mean ±SEM. * denotes significant difference versus control (p=0.013). n=13 rings from 3 patients.

3.3.3 Calcium Imaging

To confirm that tyramine was not an agonist at the α-adrenoceptors present on the RASMC, cultured RASMC loaded with the calcium sensitive dye Fluo 4 were stimulated with tyramine and responses compared with NA. The total change in fluorescence was measured over 30 seconds to determine the net effect on calcium. There was no significant increase in calcium fluorescence on addition of 1mM tyramine compared with the addition of buffer ($p=0.3014$). However there was a significant increase in fluorescence on addition of 200μM NA ($p<0.01$) and 200nM angiotensin II ($p<0.01$) (see figure 3.9).

n=3 batches. 12 wells from each batch for each agonist

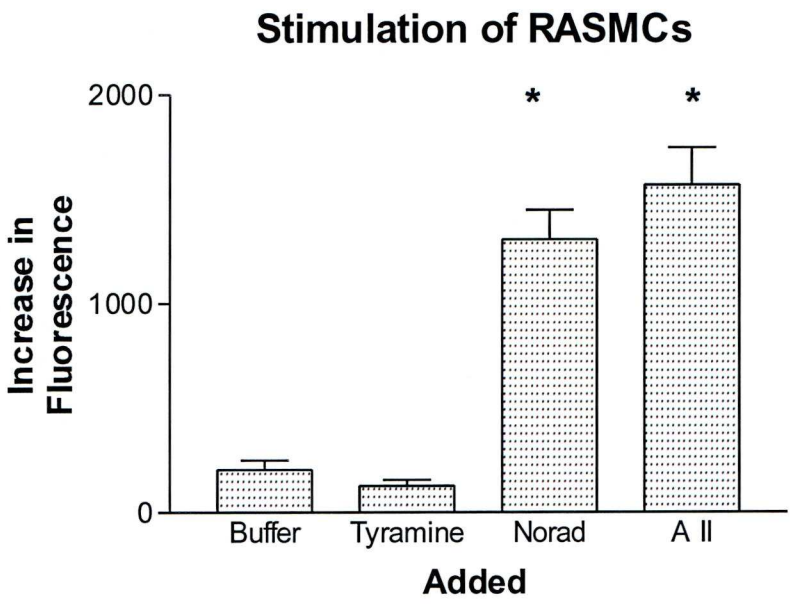

Figure 3.9 - Lack of Stimulation of RASMC's with tyramine. Bar chart showing increase in fluorescence on addition of buffer, 1mM tyramine, 20μM noradrenaline and 200nM angiotensin II. Bars represent mean ±SEM. * denotes significant increase in fluorescence.

3.4 Discussion

Vasospasm is easy to explain in the immediate post-operative period following surgical trauma and when raised levels of vasoconstrictors are present. However, spasm may occur many weeks following surgery (34;40;49;57;58;196) when one would have expected any effects of surgery to have disappeared and levels of circulating vasoconstrictors to have returned to normal.

It has been demonstrated in saphenous vein grafts that there is a decreased response to tyramine one week following implantation and there is a decrease in NA content (166). It has been suggested that these results are due to denervation The concept of denervation is further supported by the lack of responsiveness of vein grafts to electrical stimulation (194). There is no reason to suggest that a similar process does not occur in the RA. With its greater smooth muscle content when compared to the saphenous vein (197), increased contractility (198) and its increased sensitivity to catecholamines the RA would be more prone to contraction stimulated by the release of NA from degenerating nerve endings.

We investigated the hypothesis that the degeneration of nerve endings in the vessel wall could potentially release significant amounts of NA and hence cause spasm. Tyramine, a naturally occurring monoamine gets taken up into the nerve terminal via Uptake 1 (the neuronal reuptake system for NA) and then displaces NA from the storage vesicles, allowing it to leave the nerve terminal and stimulate the α adrenoceptors. Addition of tyramine to human RA rings caused a significant increase in tension in all rings tested that was equivalent to a maximum concentration

NA response. This effect was blocked by pre-treatment with the irreversible α adrenergic antagonist PhB, demonstrating that the response to tyramine was mediated by the α adrenoceptors present on the smooth muscle. However tyramine itself did not cause a rise in intracellular calcium in cultured RASMC demonstrating that it was not tyramine itself that caused the contraction. This suggests that nerve endings in the vessel wall have the potential to release significant local concentrations of NA to cause an increase in tension of the RA ring. This is similar to that seen by others in the saphenous vein (166).

After relaxation with GTN and washing off the GTN, the rings failed to respond to a second dose of tyramine. The failure of a tissue to respond to a second addition of tyramine is well described and termed tachyphylaxis. The exact mechanism of tyramine tachyphylaxis is uncertain (199). The classical hypothesis is that tyramine causes a rapid depletion of NA and further administration of tyramine does not elicit a response as the NA content of the nerve endings is no longer sufficient to cause a contraction (200). Various groups have shown that tyramine only partially depletes some tissues of NA *in vivo* (199;201;202) and *in vitro* (199;203), while others have shown that it depletes the majority of NA from nerve endings (204;205). Possible explanations for these differences may be the presence of tyramine resistant pools of NA in some tissues or species, or the ability of various tissues to reuptake or synthesise new NA. It has been demonstrated that tyramine tachyphylaxis could be prevented in rat hearts by perfusing them with tyrosine, a precursor for NA (201). Others claim a response will not occur if the NA content of the nerve ending has been depleted by greater than 75% (202). After treating the rings with a high dose of NA following tyramine administration we were then able to prevent tyramine

induced tachyphylaxis. This is presumably because the nerve endings are able to reuptake the excess NA and replenish their stores.

The possibility that the failure of the rings to respond to the second dose of tyramine was due to desensitisation of the α adrenoceptors was excluded, as all the rings responded to stimulation with NA following tyramine addition and relaxation.

He and Yang have demonstrated that the RA has a dominance of α_1 adrenoceptor function but post-junctional α_2 adrenoceptors are present (76). Post-junctional α adrenoceptors are composed of α_1 and α_2 subtypes. The contribution of α_2 adrenoceptor to drug induced contraction depends on particular blood vessels and species, as well as the diameter of the blood vessel (206;207). In the human, RA drug induced contraction is via both α_1 and α_2 subtypes (76). It is unknown which subtype of adrenoceptors mediate neuronal sympathetic vasoconstriction in the human RA. Medgett and Langer (208) reported that α_2 adrenoceptors do not mediate neuronal sympathetic vasoconstriction in the cat middle cerebral artery as they are located extra-junctionally. However, more recent reports have shown that postsynaptic α_2-adrenoceptors can be involved in the vasoconstrictor response to sympathetic nerve stimulation to a significant (209) or even predominant level (76;83). These experiments confirmed that the tyramine evoked contraction in the RA is via α adrenoceptors as it is blocked by the irreversible α adrenoreceptor antagonist, PhB. Further work is needed to determine whether this response is via the α_1 or α_2 subtype of adrenoceptor.

It has been shown that saphenous vein grafts show an altered vascular reactivity (194;210). Soliman *et al.* have demonstrated that there is an enhanced sensitivity in

grafted vessels to NA at a week following grafting, as the NA dose response curve is shifted to the left (166). This enhanced sensitivity may be due to denervation as it is seen in vein grafts that are placed in the arterial or venous systems (194). Despite there being a super-sensitivity to NA in saphenous vein grafts, the maximal contraction of grafted vessels to NA is reduced when grafted in the arterial system (166;194;210). This has also been shown in the saphenous vein following high pressure distension (211). Morphological changes such as mechanical deformation of vascular smooth muscle (212), and structural reorganisation of the contractile elements within the vessel wall (213) could explain the reduced maximal contraction. This decrease in maximal contractility does not occur in the saphenous vein when it is grafted into the venous system. (194). The morphological changes seen in venous grafts placed in the arterial system have not been demonstrated in arterial grafts in the postoperative period, therefore the maximal contractile response in these arteries may not be decreased, and in fact may be enhanced due to the any super-sensitivity present.

Degenerating nerve endings may be a potential cause of spasm *in vivo*, and would explain why spasm is still seen after levels of circulating vasoconstrictors have returned to normal following surgery, and any effect of handling during the operation resolved. It was shown in these experiments that PhB prevented contraction of the RA due to tyramine evoked release of NA. A way of preventing spasm due to degenerating nerve endings releasing NA for the first 48 hours post-operatively is by the topical application of PhB at the time of surgery (143). However there will be regeneration of α receptors over the 48 hours following surgery and the effects of PhB will not be present after this time. Tyramine could be used to degranulate nerve endings at the time of surgery prior to grafting but we believe the nerve endings may

reuptake NA, especially as circulating NA levels are raised in the post-operative period (165), or synthesise more NA for a release at a later date making this treatment ineffective.

Three possible drugs that could alter the release of NA from nerve endings, reserpine, guanethidine and 6 hydroxydopamine, were investigated. Reserpine inhibits the amine uptake process in the vesicle membrane and thereby allows leakage of NA into the cytoplasm where it is broken down by neuronal MAO. Furthermore, since vesicular dopamine uptake is inhibited, NA synthesis is impaired. NA depletion is accelerated by action potential activity in the neurone. Noradrenergic transmission fails when NA content is reduced to 25% of normal. When large doses of reserpine are used, recovery of neuronal function depends on synthesis of new vesicles and their transport to the axon terminals which takes approximately 10 days. Guanethidine impairs transmission at the neuroeffector junction

Of these drugs, reserpine abolished the response of the RA to tyramine. This is similar to the effects of reserpine on the rat heart. One would expect the effects of reserpine to last at least 10 days for reasons stated above; the effects may however be permanent if the nerves are no longer functional. 6-Hydroxydopamine failed to abolish the response of the RA to tyramine. This is not what was expected as 6-hydroxydopamine works in a similar way to reserpine. It may be that a higher concentration or longer incubation period was required. Further experiments are

needed to investigate this. Guanethidine failed to deplete the nerve endings of NA. This may well be due to the denervated nature of the nerve endings.

In conclusion nerve endings in the wall of the RA have the potential to degranulate and cause contraction of the RA rings. This may be one of the mechanisms that cause spasm in the RA. Spasm could be prevented by destroying the NA vesicles with reserpine at the time of operation prior to grafting, or degranulating them with tyramine and reversing the contraction with GTN. A combination of the two methods may be necessary.

Chapter 4

Regeneration of α–Adrenoceptors

4.1 Introduction

PhB has been recommended to prevent spasm in RA conduits for CABG (150). PhB binds irreversibly to the α_1-adrenoceptor, the major adrenoceptor on the human RA (76). Consequently, the vascular smooth muscle must regenerate new receptors *de novo*, before the response to NA is restored (167). The action of PhB persists for several days (144), however, in smooth muscle cells cultured in the presence of angiotensin II, adrenoceptor regeneration occurs to levels beyond that originally present (168).

The explanation for these findings could be due to the fact that angiotensin II plays a role as a growth modulator in vascular smooth muscle (214). Angiotensin II has been shown to increase vascular smooth muscle cell DNA synthesis *in vivo* and *in vitro* (215-217). ACE inhibitors, which inhibit the formation of angiotensin II, have been shown to prevent the development of neointimal hyperplasia after balloon injury in the rat (218). Activation of protein kinase C leads to a marked induction of the α_1-adrenoceptor gene (219). Angiotensin II is known to activate protein kinase C in vascular smooth muscle via the stimulation of phospholipase C (220).

Since angiotensin II levels are raised following cardiopulmonary bypass (165), there may be an overshoot in adrenoceptor regeneration in the RA if it is pre-treated with PhB prior to grafting. If receptor regeneration occurs and leads to 'sensitisation' of the artery PhB would therefore prevent spasm in the initial postoperative period, but then the RA would become more sensitive to catecholamines and hence prone to spasm later.

To demonstrate if this expected overshoot in adrenoceptor sensitivity occurs the sensitivity of cultured RASMC $[Ca^{2+}]_c$ responses to NA stimulation will be examined after a period of pre-treatment and recovery from PhB. RASMC will be grown on five 96 well plates. Following treatment of half of each plate with 1μM PhB for 1 hour (sufficient to completely block the α-adrenoceptors of the RA (144), concentration response curves will be obtained from cultured cells over a period of five days to measure the regeneration of functional adrenoceptors. The experiments will be repeated with the cells incubated in the presence of angiotensin II, ET I, vasopressin or NA after the treatment with PhB.

The experiments will then be repeated in an organ culture model to confirm the results from cultured cells.

4.2 Methods and Materials

4.2.1 Calcium Imaging

RASMC were cultured as described in chapter 2. When confluent in a $75cm^3$ tissue culture flask they were reseeded onto five 96 well plates. The cells were grown on the 96 well plates to confluence. Once confluent, rows 1-6 of all 5 of the plates were treated with 1μM PhB in DMEM containing antibiotics and 20% FBS for 1 hour at 37°C. Rows 7-12 were just treated with the DMEM. After the hour the wells were aspirated and culture media added to four of the plates. The remaining plate was loaded with fluo 4-AM dye and calcium imaging carried out as described in chapter 2. The remaining four 96 well plates underwent media change daily till imaged. One plate was imaged on each of the following days. Three plates, from three different cell lines were imaged for the day of treatment and the 4 subsequent days. Parallel studies were also carried out with the cells incubated in the presence of 100nM angiotensin II, 100nM ET-1, 100nM vasopressin and 10μM NA to measure whether receptor regeneration is affected. Culture media was changed every 24 hours to maintain levels of vasoconstrictors.

The following additions were carried out

Row	Addition
A	Buffer
B	0.1μM Noradrenaline
C	1μM Noradrenaline
D	2μM Noradrenaline
E	4μM Noradrenaline
F	10μM Noradrenaline
G	100μM Noradrenaline
H	100nM Angiotensin II

Each experiment was repeated with 3 different batches of cells.

4.2.2 Organ Culture Study

Distal segments of RA, pre-treated with 6mM PhB in theatre, were divided into rings and used for experiments on that day (Day 0) or cultured as described in chapter 2. On consecutive days a RA ring was mounted in the organ bath and pre-tensioned as described in chapter 2. Responses to 90mM KCl, increasing concentrations of NA (0.1μM, 1μM, 2μM, 4μM, 10 μM and 100 μM) and 100nM vasopressin were recorded. If the ring did not respond to KCl then another ring was selected and the process repeated on the same day.

4.3 Results

All cells were confirmed as being smooth muscle cells as described in chapter 2.

4.3.1 Calcium Imaging

Initially the cells were incubated with 6mM PhB to replicate the concentrations used in clinical practice (193). However after treatment, the 96 well plate was viewed under the microscope and the cells were found to have lifted from the bottom of each well. This was thought to have been due to the acidic nature of PhB. The effect of increasing concentrations of PhB on blood and Saline pH was investigated (See figure 4.1). It was shown that the pH of saline dropped significantly on addition of 100μM PhB.

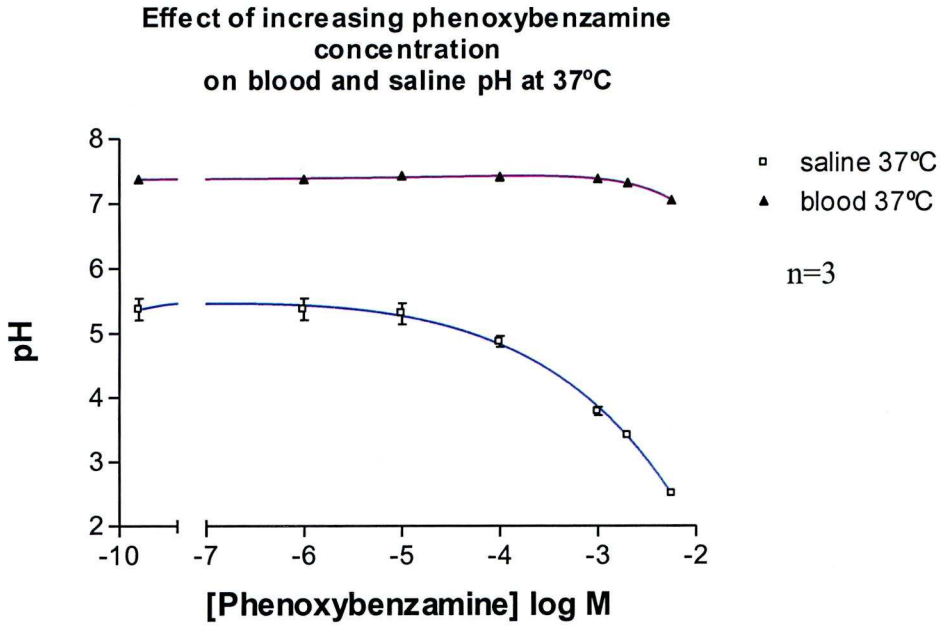

Figure 4.1- Effect of increasing concentrations of PhB on blood and Saline pH. Increasing concentrations of PhB was added to 50 ml human blood containing 5000 i.u heparin or 145mM saline at 37^{0}C and the pH measured. Mean pH and SEM are plotted.

It has previously been shown by others that 1μM PhB caused complete inhibition of the response of RA rings to NA (221). We therefore adopted this concentration for all further experiments and the problems with the cells lifting were resolved.

RASMC cultured from 11 different patients were used for this study. Three batches of RASMC from a further 3 patients were not used as they developed a fungal infection. Patient characteristics are shown in table 4.1.

Mean age (years)	67
Sex ratio (Male:Female)	7:4
Risk Factors (n)	
Current Smokers	0
Ex-smokers	7
Non-smokers	4
Hypertension	6
Diabetes mellitus	1
Peripheral Vascular disease	2
Previous CVA or TIA	0
Preoperative Medication (n)	
β blockers	7
Calcium channel blockers	5
ACE inhibitors	3
Nitrates	9
Potassium channel openers	3

Table 4.1 – Patient characteristics (n=11)

On day 0, none of the RASMC treated with PhB showed a significant increase in peak height of calcium-sensitive fluo-4 fluorescence on addition of NA. However, all the cells that were not treated with PhB showed an increase in calcium fluorescence with addition of NA, EC_{50} 2.49 μM (95% CI 0.90-6.96 μM) (figure 4.2a). There was no significant difference between those treated with PhB and the untreated cells in response to angiotensin II addition (Table 4.2).

On day 1 the PhB treated cells had a similar EC_{50} to the untreated cells, EC_{50} 3.30 μM vs 2.67μM but the maximal increase in fluorescence was significantly less (figure 4.2b). The response to angiotensin II remained similar in the untreated and PhB treated cells on this and all subsequent days (Table 4.3).

On day 2, and the subsequent 2 days the concentration response curves were similar in the treated and untreated groups (figure 4.2c,4.2d,4.2e,4.2f). Table 4.2 shows the EC_{50} of NA on each of the days.

Similar responses were seen when the cells were cultured in 100nM angiotensin II (table 4.4 and figure 4.3), 100nM ET-1 (table 4.5 and figure 4.4), 100 nM vasopressin (table 5.6 and figure 5.5), and 10μM NA (table 6.7 and figure 6.6).

Day	PhB Treated	PhB Untreated	Significance
0	1883.28±33.21	1884.13±30.59	NS
1	1904.778±26.03	1879.87±30.23	NS
2	1862.28±39.59	1873±30.52	NS
3	1937.22±38.23	1878.73±31.24	NS
4	1973.5±26.7	1898±35.36	NS

Table 4.2 – Peak increase in calcium fluorescence in response to addition of 100nM angiotensin II in 1μM PhB treated and untreated RASMC's. n=18 wells for each group in total (6 wells from 3 batches). Values are expressed as mean ±SEM. Comparison is made using a paired student's t test.

RASMCs incubated with no vasoconstrictor

Day	PhB treated Noradrenaline EC_{50} (μM) (95% CI)	PhB Untreated Noradrenaline EC_{50} (μM) (95% CI)
0	No response	2.49 (0.90-6.96)
1	3.30 (0.938-11.12)	2.92 (1.26-6.78)
2	3.30 (1.51-7.20)	2.62 (0.73-9.73)
3	3.89 (2.76-5.47)	2.41 (0.70-8.37)
4	3.37 (1.14-9.94)	2.80 (1.01-7.13)

Table 4.3 – Shows EC_{50} NA (μM) and 95% confidence intervals in 1μM PhB treated and untreated RASMC on Days 0-4. EC_{50} were calculated using Graphpad Prism version 3.02. Concentration response curves were constructed by adding 0.1μM, 1μM, 2μM, 4μM, 10μM and 100μM NA to 6 wells on each of 3 batches of RASMC

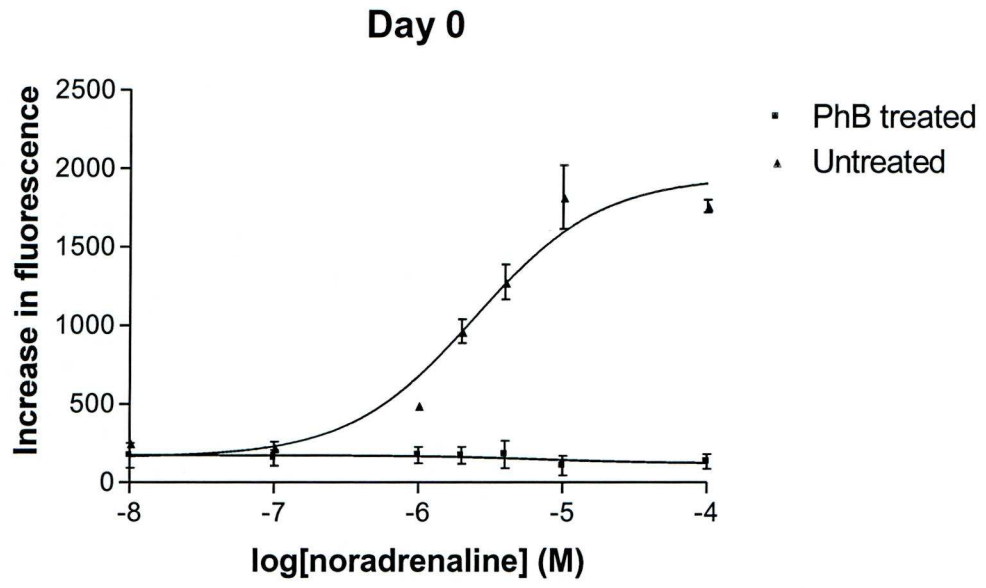

Figure 4.2a – Day 0

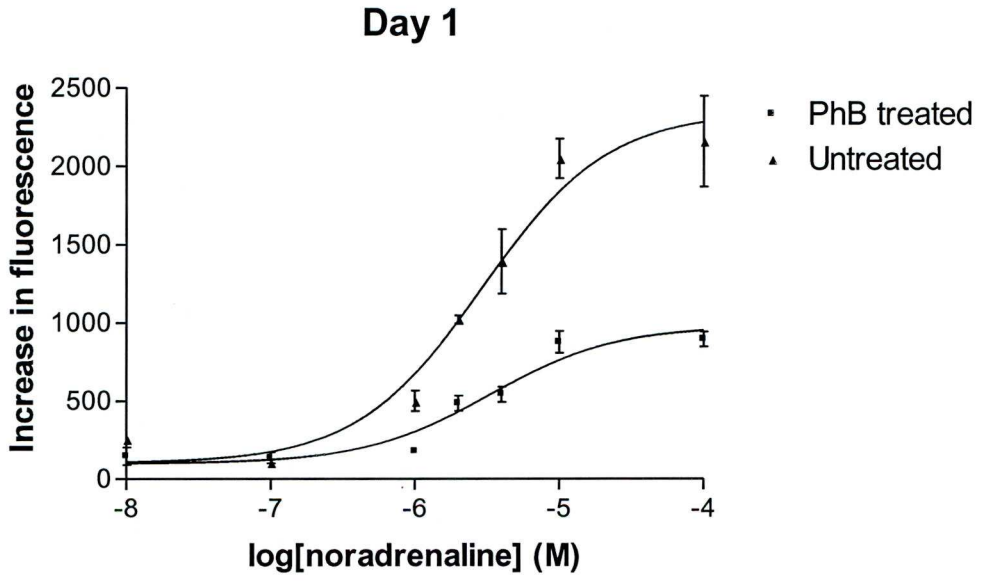

Figure 4.2b – Day 1

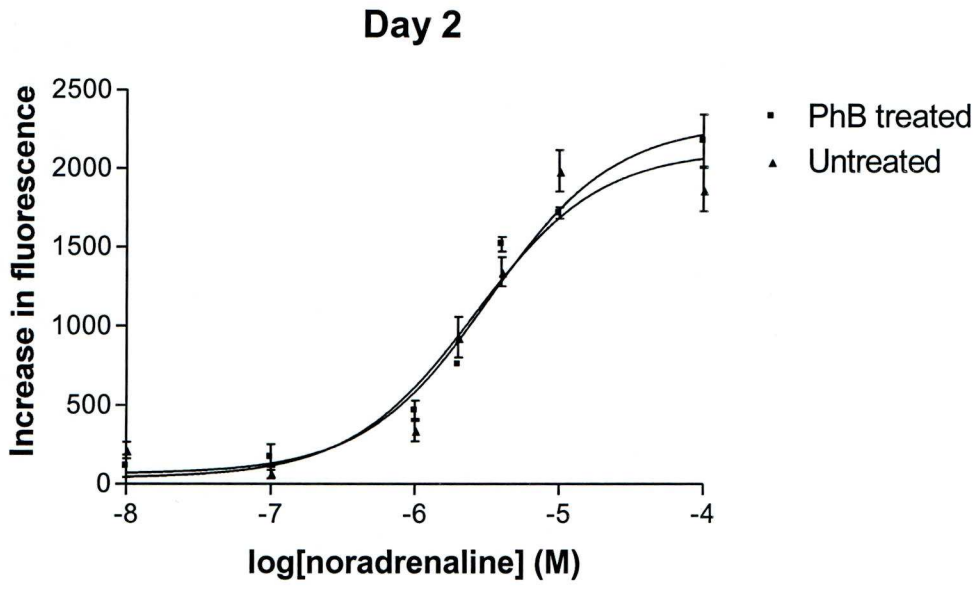

Figure 4.2c – Day 2

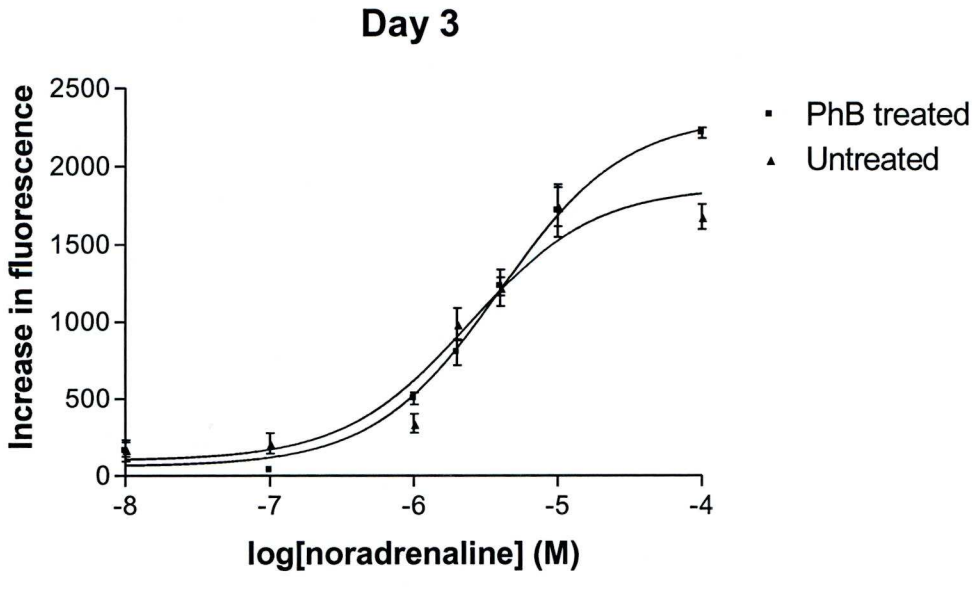

Figure 4.2d – Day 3

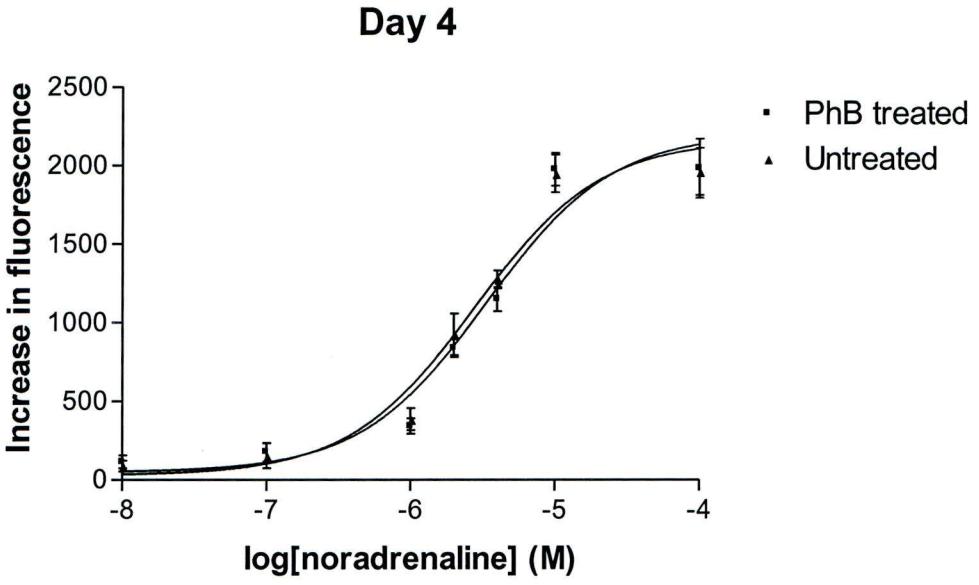

Figure 4.2e – Day 4

Figure 4.2 – Concentration response curves for NA in 1μM PhB treated and untreated human RASMC on days 0-4. Concentration response curves were produced using Graphpad Prism version 3.02. They were constructed by adding 0.1μM, 1μM, 2μM, 4μM, 10μM or 100μM NA to 6 wells from each of 3 batches of RASMC. Mean increase in fluorescence ± SEM are plotted. Figure 4.2a represents 2 hours following PhB treatment, 4.2b 1 days after, 4.2c 2 days after, 4.2d 3 days after, 4.2e 4 days after

RASMC incubated with 100nM Angiotensin II

Day	PhB treated Noradrenaline EC_{50} (μM) (95% CI)	PhB Untreated Noradrenaline EC_{50} (μM) (95% CI)
0	No response	2.83 (1.43-5.63)
1	3.06 (1.92-4.85)	3.05 (1.73-5.38))
2	3.44 (2.18-5.41)	2.26 (1.03-4.80)
3	3.50 (1.63-7.59)	3.27 (2.39-4.48)
4	3.16 (1.64-6.12)	2.56 (1.74-3.78)

Table 4.4 – Shows EC_{50} NA (μM) and 95% confidence intervals in 1μM PhB treated and untreated RASMC incubated in the presence of 100nM angiotensin II on Days 0-4. EC_{50} were calculated using Graphpad Prism version 3.02. Concentration response curves were constructed by adding 0.1μM, 1μM, 2μM, 4μM, 10μM and 100μM NA to 6 wells on each of 3 batches of RASMC

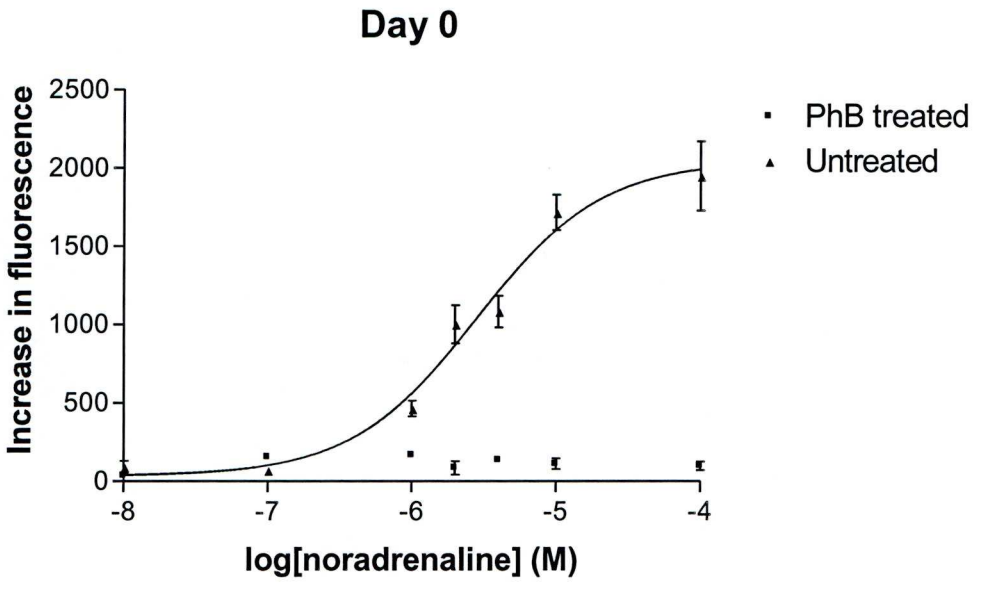

Figure 4.3a – Day 0

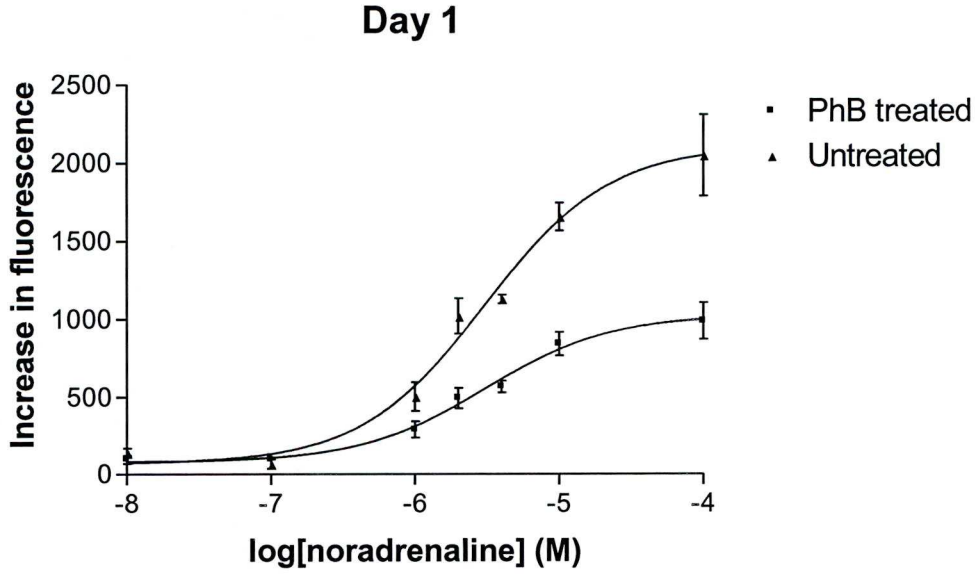

Figure 4.3b – Day 1

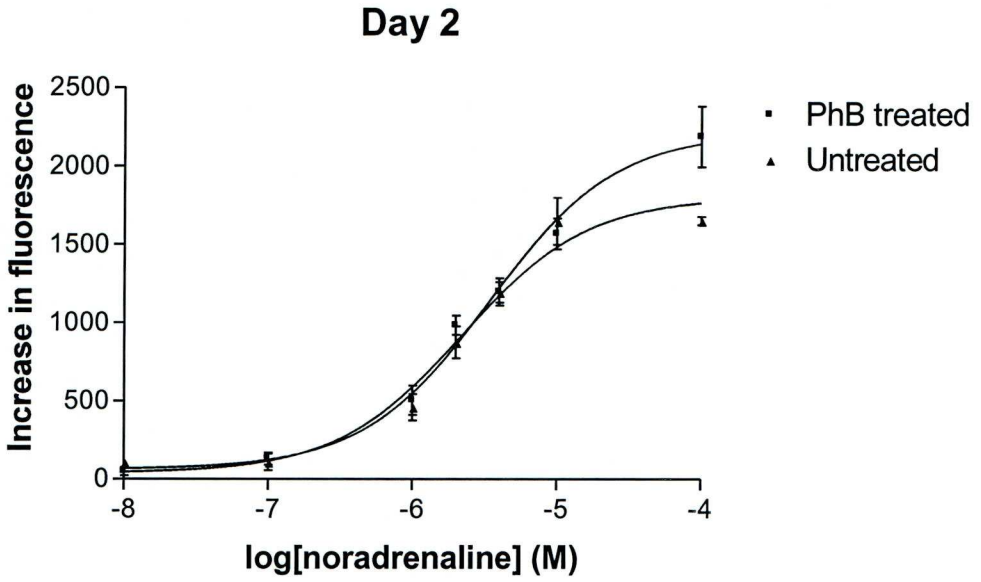

Figure 4.3c – Day 2

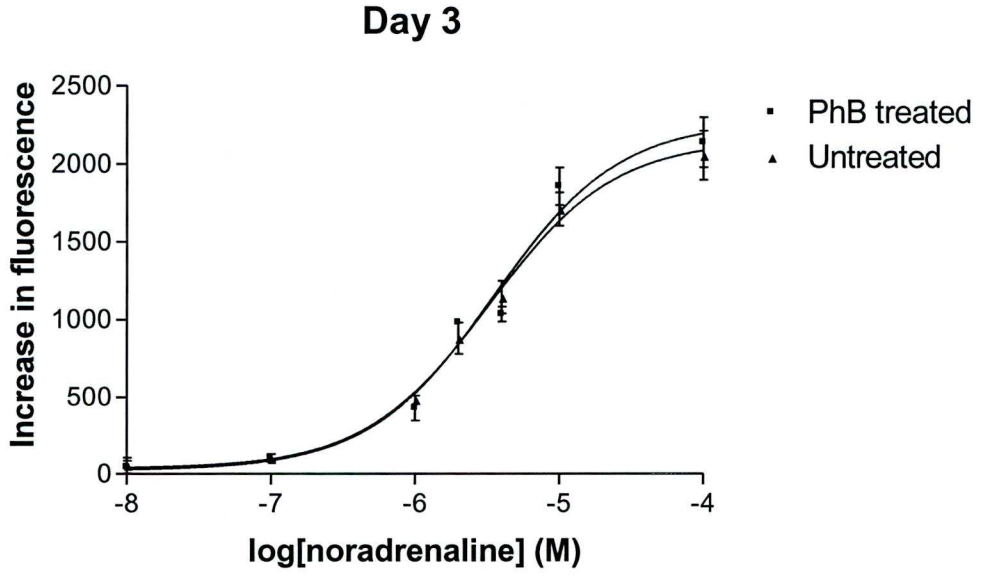

Figure 4.3d – Day 3

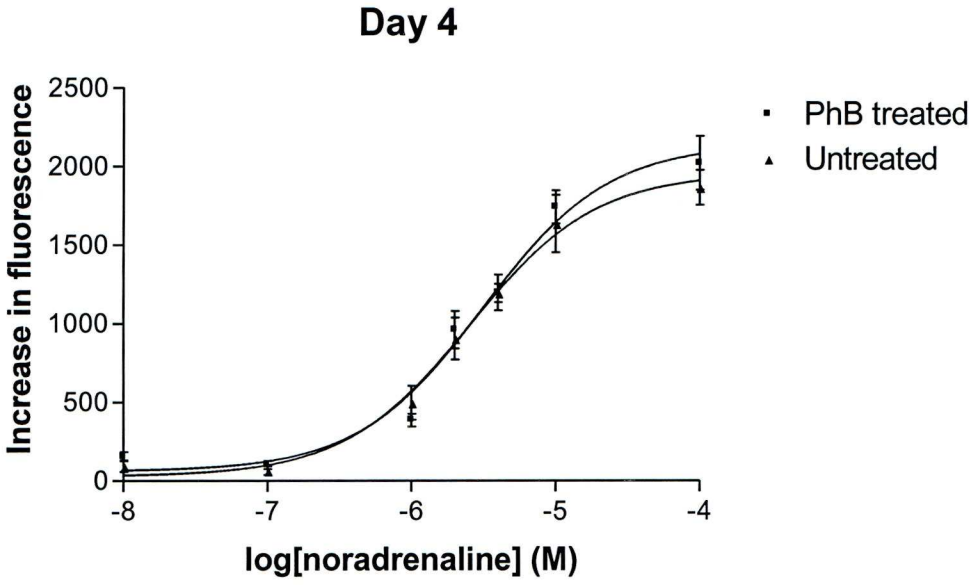

Figure 4.3e – Day 4

Figure 4.3 – Concentration response curves for NA in 1μM PhB treated and untreated human RASMC incubated in 100nM angiotensin II on days 0-4. Concentration response curves were produced using Graphpad Prism version 3.02. They were constructed by adding 0.1μM, 1μM, 2μM, 4μM, 10μM or 100μM NA to 6 wells from each of 3 batches of RASMC. Mean increase in fluorescence ± SEM are plotted. Due to scatter of points in the PhB treated group on day 0 no curve could be constructed. Figure 4.3a represents 2 hours following PhB treatment, 4.3b 1 days after, 4.3c 2 days after, 4.3d 3 days after, 4.3e 4 days after

RASMC incubated with 100nM Endothelin I

Day	PhB treated Noradrenaline EC_{50} (μM) (95%CI)	PhB Untreated Noradrenaline EC_{50} (μM) (95% CI)
0	Not response	2.42 (0.93-6.23)
1	0.40 (0.0003-7.06)	2.64 (1.01-6.89)
2	2.95 (1.15-7.60)	3.23 (1.68-6.23)
3	2.47 (1.41-4.34)	3.30 (2.30-4.76)
4	3.51 (1.75-7.16)	3.92 (2.22-6.95)

Table 4.5 – Shows EC_{50} NA (μM) in 1μM PhB treated and untreated RASMC incubated in 100nM endothelin I on Days 0-4. EC_{50} were calculated using Graphpad Prism version 3.02. Concentration response curves were constructed by adding 0.1μM, 1μM, 2μM, 4μM, 10μM and 100μM NA to 6 wells on each of 3 batches of RASMC

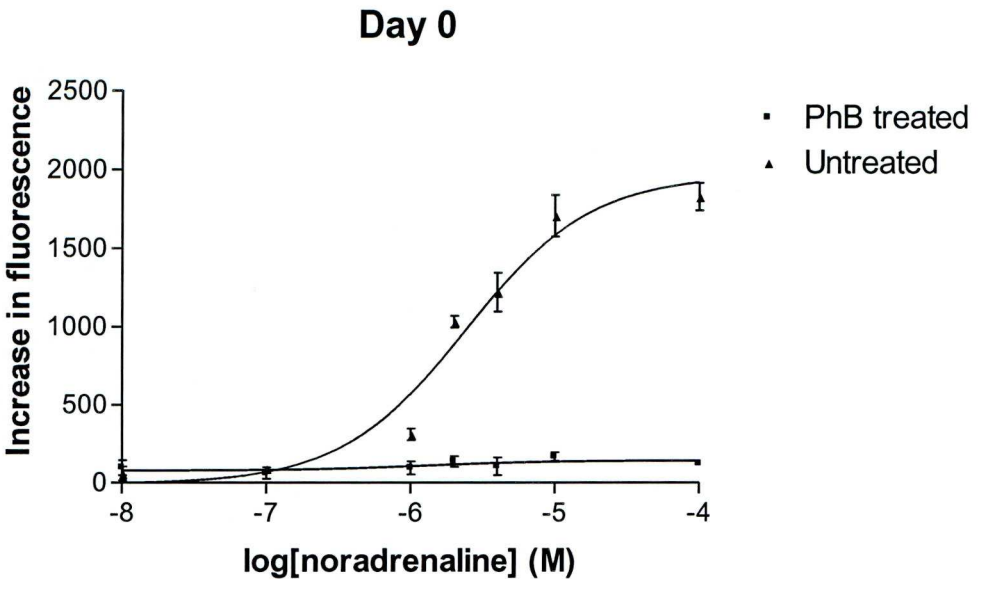

Figure 4.4a – Day 0

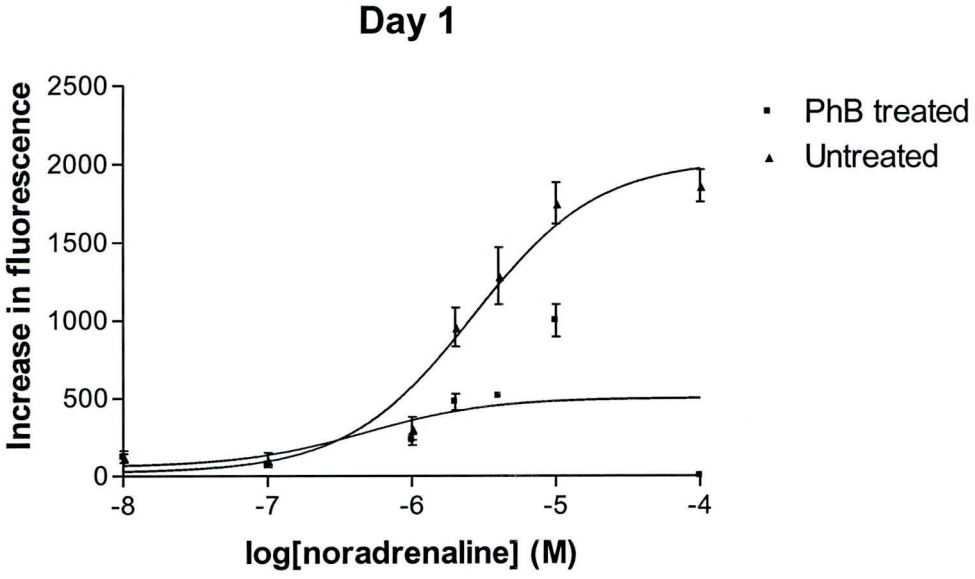

Figure 4.4b – Day 1

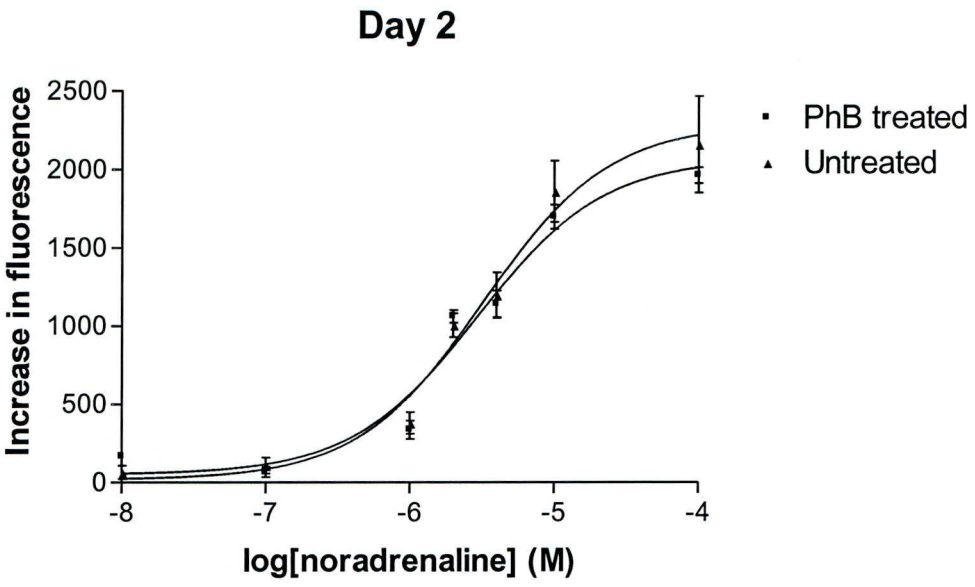

Figure 4.4c – Day 2

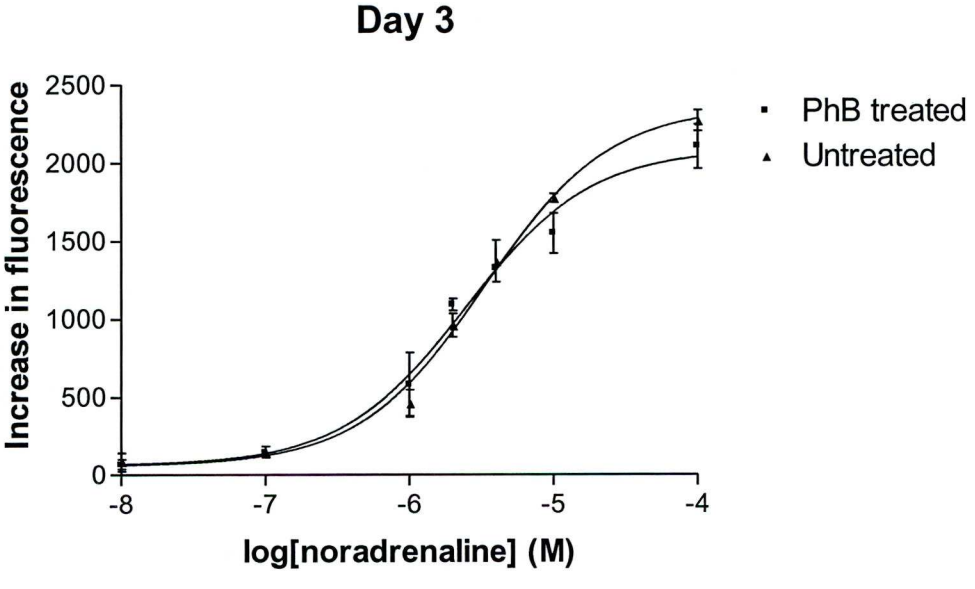

Figure 4.4d – Day 3

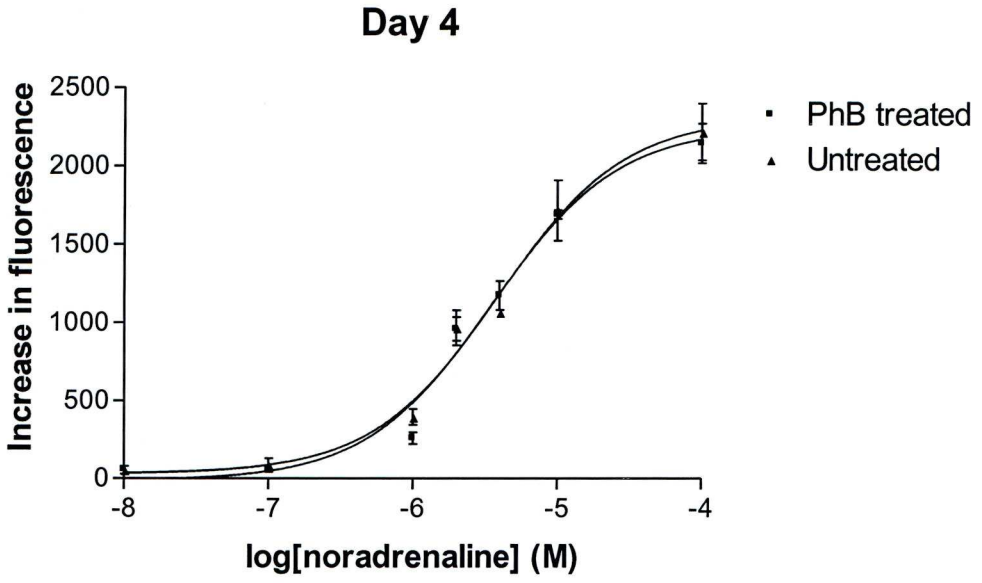

Figure 4.4e – Day 4

Figure 4.4 – Concentration response curves for NA in 1μM PhB treated and untreated human RASMC incubated in 100nM endothelin I on days 0-4. Concentration response curves were produced using Graphpad Prism version 3.02. They were constructed by adding 0.1μM, 1μM, 2μM, 4μM, 10μM or 100μM NA to 6 wells from each of 3 batches of RASMC. Mean increase in fluorescence ± SEM are plotted. Figure 4.2a represents 2 hours following PhB treatment, 4.2b 1 days after, 4.2c 2 days after, 4.2d 3 days after, 4.2e 4 days after

RASMC incubated with 100nM Vasopressin

Day	PhB treated Noradrenaline EC_{50} (μM) (95% CI)	PhB Untreated Noradrenaline EC_{50} (μM) (95%CI)
0	No response	2.36 (0.94-6.23)
1	1.58 (0.003-7.60)	2.68 (1.01-6.89)
2	2.96 (1.15-7.60)	3.24 (1.68-6.23)
3	2.47 (1.41-4.34)	3.33 (2.33-4.76)
4	3.54 (0.75-7.16)	3.92 (2.22-6.95)

Table 4.6 – Shows EC_{50} NA (μM) (95% confidence Intervals) in 1μM PhB treated and untreated RASMC incubated in 100nM vasopressin on Days 0-4. EC_{50} were calculated using Graphpad Prism version 3.02. Concentration response curves were constructed by adding 0.1μM, 1μM, 2μM, 4μM, 10μM and 100μM NA to 6 wells on each of 3 batches of RASMC

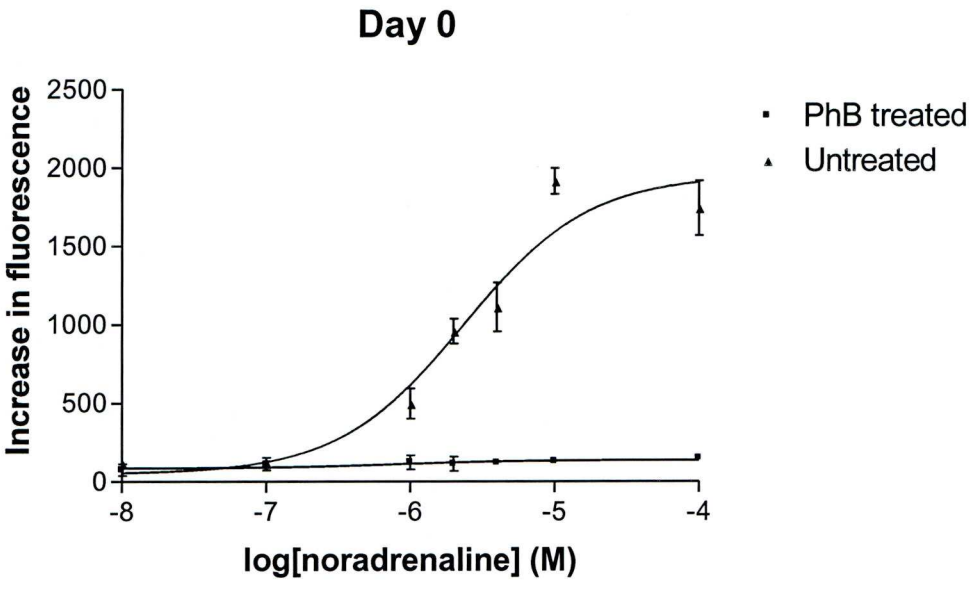

Figure 4.5a –Day 0

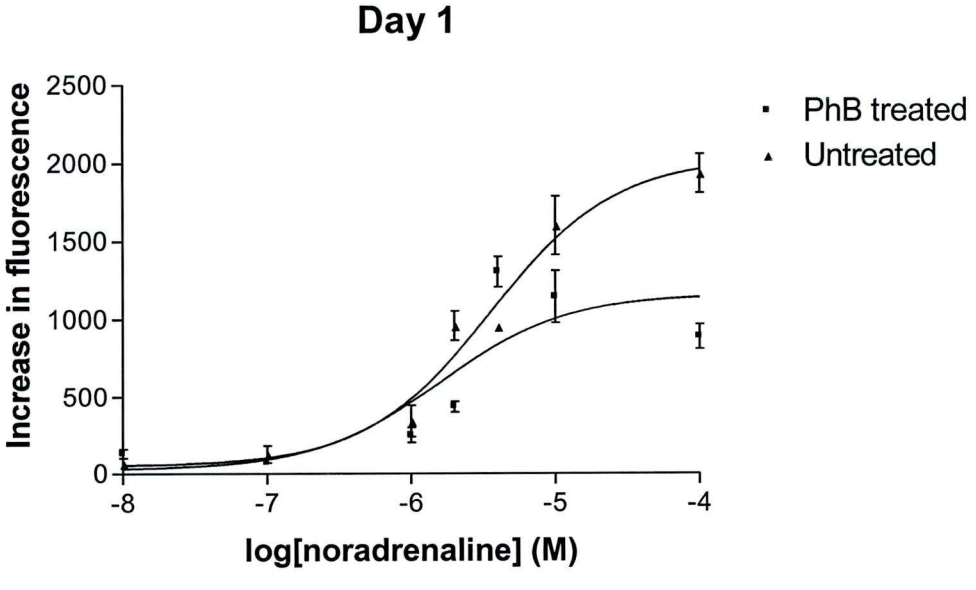

Figure 4.5b – Day 1

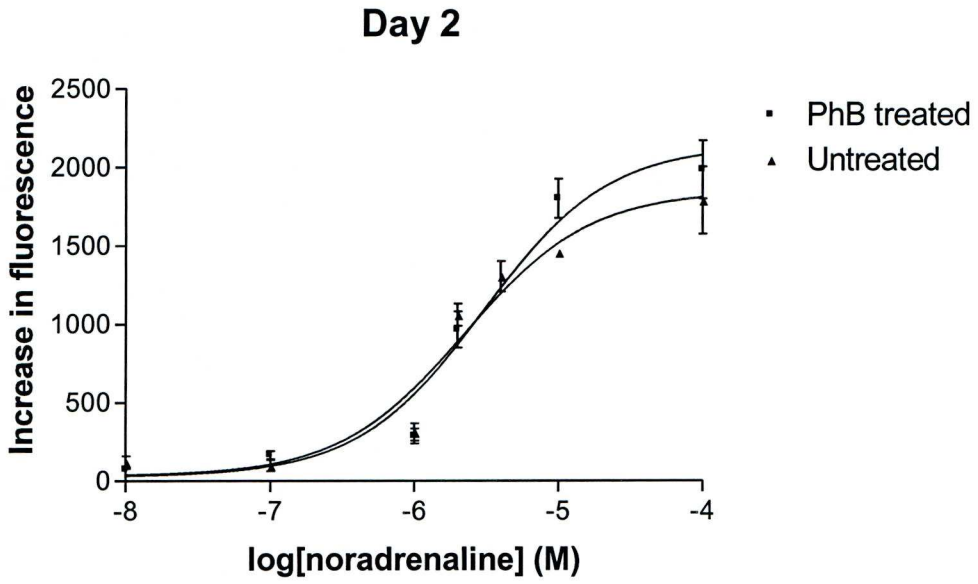

Figure 4.5c – Day 2

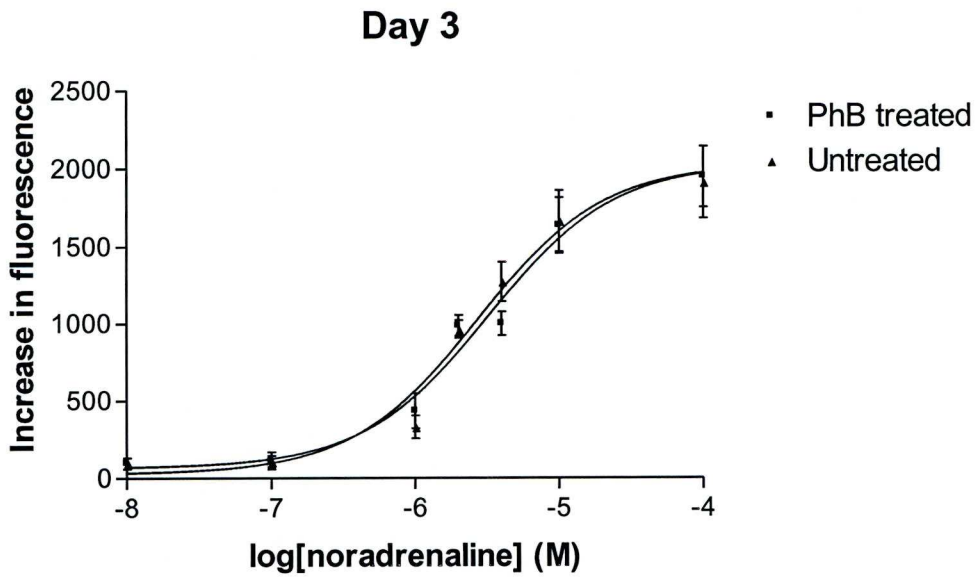

Figure 4.5d – Day 3

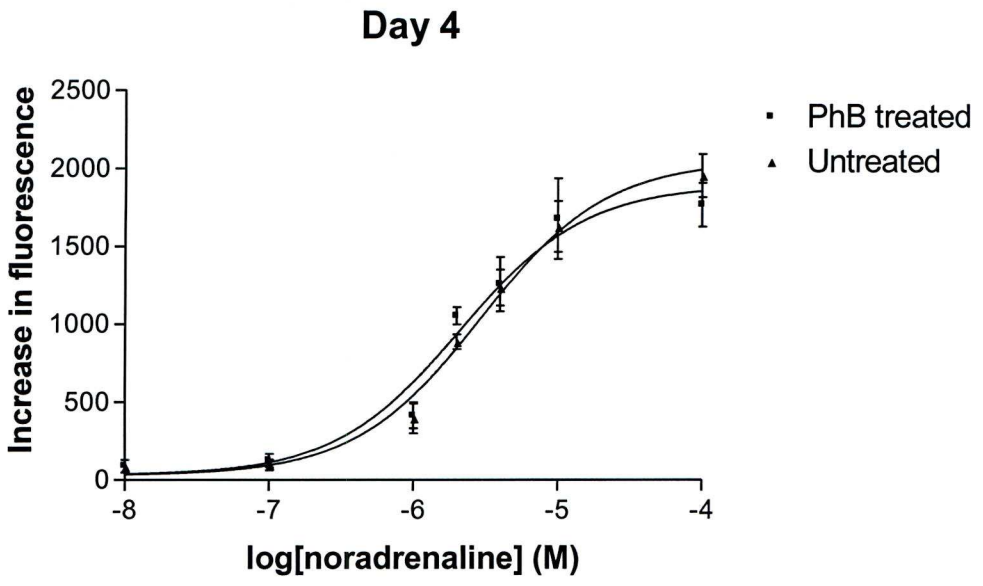

Figure 4.5e – Day 4

Figure 4.5 – Concentration response curves for NA in 1μM PhB treated and untreated human RASMC incubated in 100nM vasopressin on days 0-4. Concentration response curves were produced using Graphpad Prism version 3.02. They were constructed by adding 0.1μM, 1μM, 2μM, 4μM, 10μM or 100μM NA to 6 wells from each of 3 batches of RASMC. Mean increase in fluorescence ± SEM are plotted. Figure 4.5a represents 2 hours following PhB treatment, 4.5b 1 days after, 4.5c 2 days after, 4.5d 3 days after, 4.5e 4 days after

RASMC incubated with 10μM Noradrenaline

Day	PhB treated Noradrenaline EC_{50} (μM) (95% CI)	PhB Untreated Noradrenaline EC_{50} (μM) (95% CI)
0	No response	2.36 (0.78-7.80)
1	1.58 (0.06-36.6)	3.52 (1.52-8.18)
2	3.03 (0.79-11.59)	2.24 (0.75-6.66)
3	3.24 (1.54-6.85)	2.74 (1.26-5.94)
4	2.13 (1.00-4.98)	2.96 (1.85-4.73)

Table 4.7 – Shows EC_{50} NA(μM) (95%confidence intervals) in 1μM PhB treated and untreated RASMC incubated in 10μM NA on Days 0-4. EC_{50} were calculated using Graphpad Prism version 3.02. Concentration response curves were constructed by adding 0.1μM, 1μM, 2μM, 4μM, 10μM and 100μM NA to 6 wells on each of 3 batches of RASMC

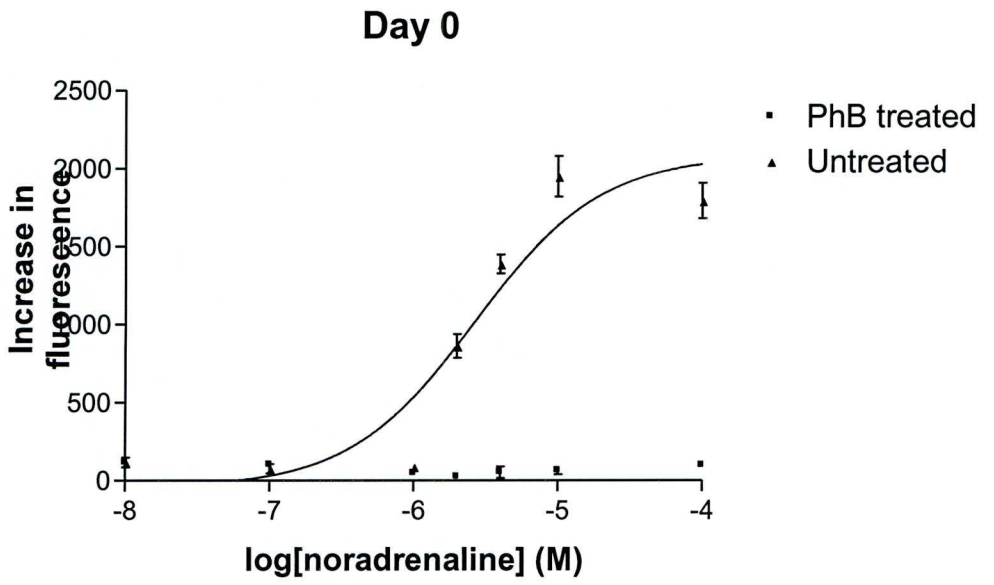

Figure 4.6a - Day 0

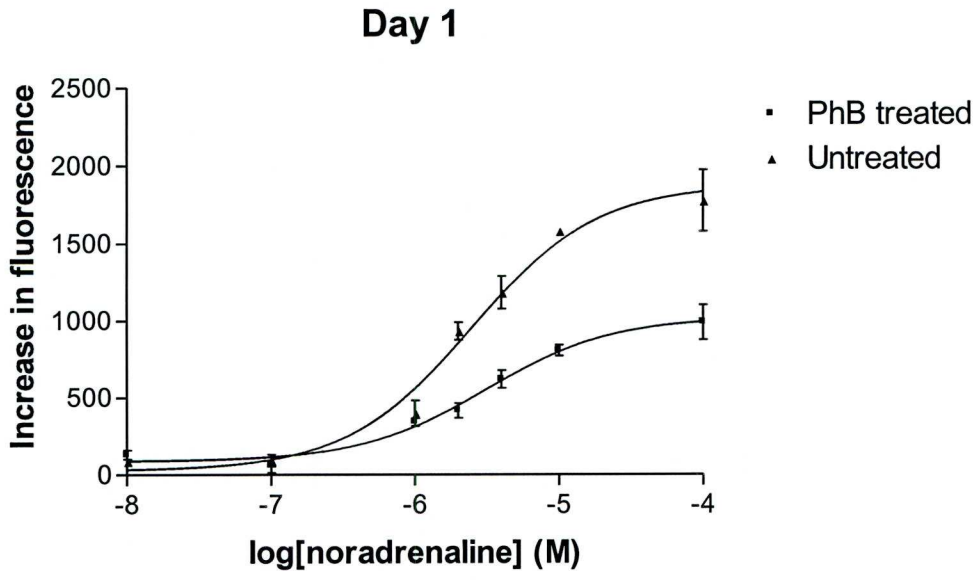

Figure 4.6b – Day 1

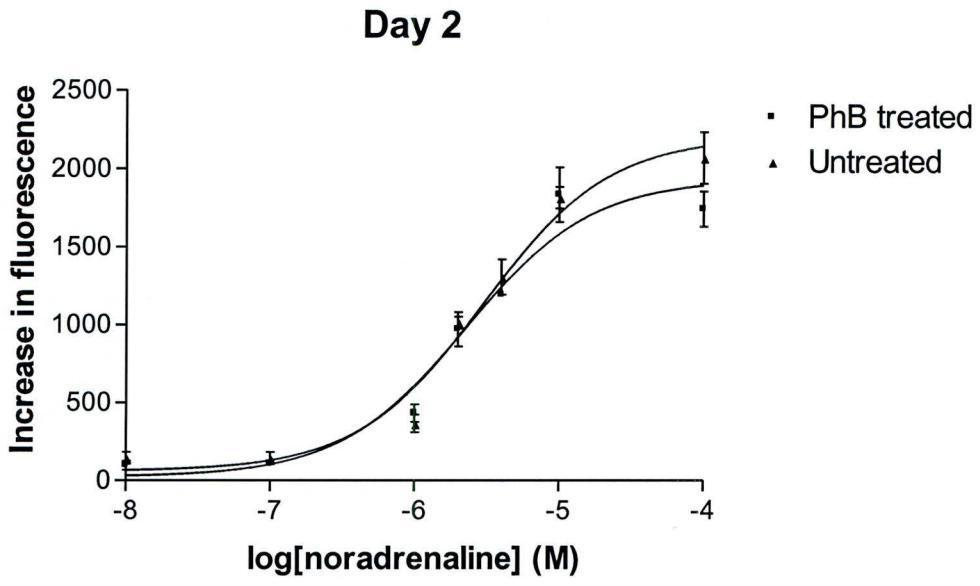

Figure 4.6c – Day 2

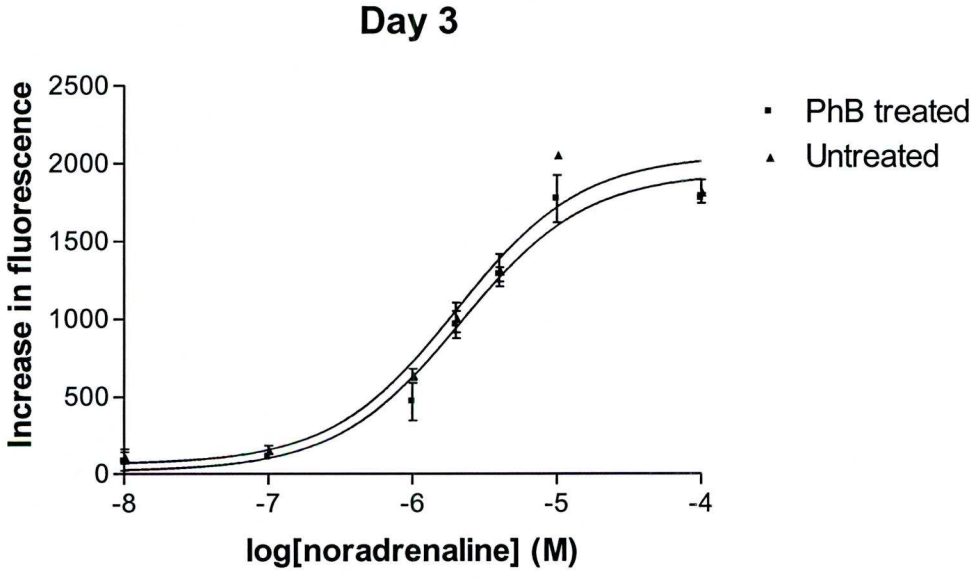

Figure 4.6d – Day3

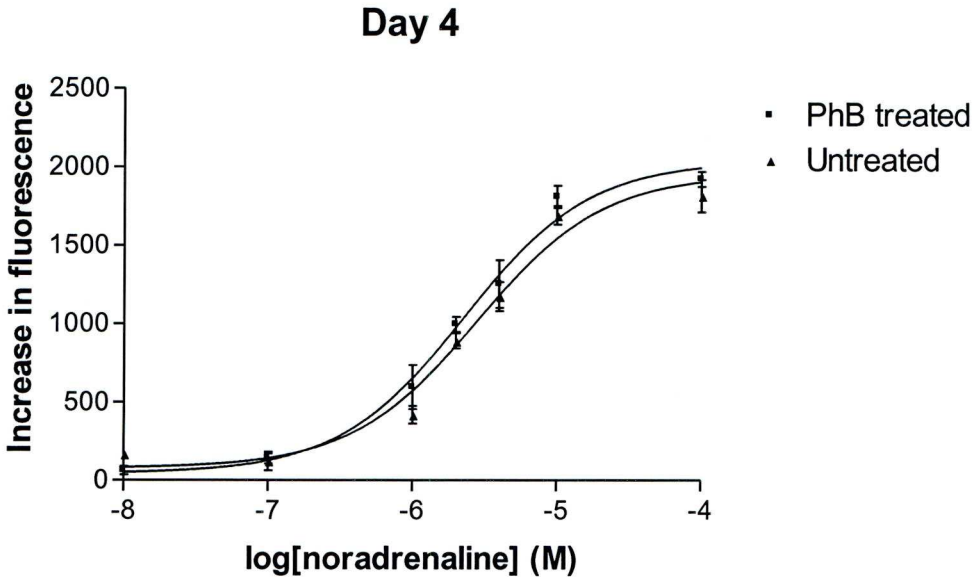

Figure 4.6 – Concentration response curves for NA in 1μM PhB treated and untreated human RASMC incubated in 10μM NA on days 0-4. Concentration response curves were produced using Graphpad Prism version 3.02. They were constructed by adding 0.1μM, 1μM, 2μM, 4μM, 10μM or 100μM NA to 6 wells from each of 3 batches of RASMC. Mean increase in fluorescence ± SEM are plotted. Figure 4.6a represents 2 hours following PhB treatment, 4.6b 1 days after, 4.6c 2 days after, 4.6d 3 days after, 4.6e 4 days after

At no point in time, in any group of experiments, was there a significant change in the EC_{50} for NA or a maximal increase in fluorescence in the PhB treated cells compared to the untreated group. This demonstrates that an overshoot in α-adrenoceptors does not occur in the human RA.

4.3.2 Organ Culture Study

For these experiments, RA rings from 11 patients were used. The patient characteristics are shown in table 4.8.

Total number of Patients	11
Mean age (years)	57
Sex ratio (Male:Female)	6:5
Risk Factors (n)	
Current Smokers	0
Ex-smokers	4
Non-smokers	7
Hypertension	8
Diabetes mellitus	2
Peripheral Vascular disease	3
Previous CVA or TIA	0
Preoperative Medication (n)	
B blockers	9
Calcium channel blockers	7
ACE inhibitors	5
Nitrates	9
Potassium channel openers	4

Table 4.8 – Patient characteristics (n=11)

There was a decrease in the initial response of the rings to 90mM KCl on day 1 compared with day 0, 3.86g ±0.5 vs. 6.03g ±0.56 (p=0.008). On day 2, only 2 out of 12 rings responded to 90mM KCl. On the 3rd day none, of the remaining 6 rings from the 2 patients whose rings responded on day 2, responded to addition of 90mM KCl (see figure 4.7).

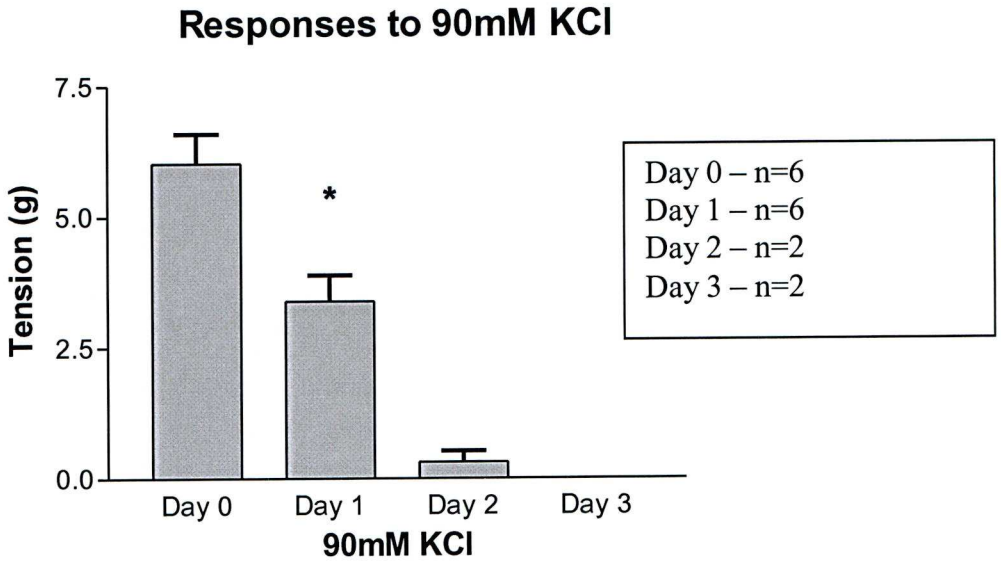

Figure 4.7 – 90mM KCl responses in 6mM PhB treated RA rings on consecutive days. Rings were incubated in DMEM at 37^{0}C under 6g tension. Bars represent mean ±SEM. * Denotes significant decrease in tension from the previous day (p=0.008)

At no time were we able to elicit a NA induced contraction in any of the rings, on any day.

Vasopressin responses followed a similar pattern to the KCl responses. All rings that responded to 90mM KCl, responded to vasopressin. None of the rings that did not respond to 90mM KCl responded to vasopressin. Although there was a significant difference in the increase in tension seen in response to vasopressin on day 0

compared to day 1 7.51g ±0.61vs 3.82g 0.24 (p=0.0005) (Figure 4.8), when expressed as a % of the KCl response, there was no significant difference 125.8%±5.7 vs. 118.49±9.54 (p=0.53) (Figure 4.9).

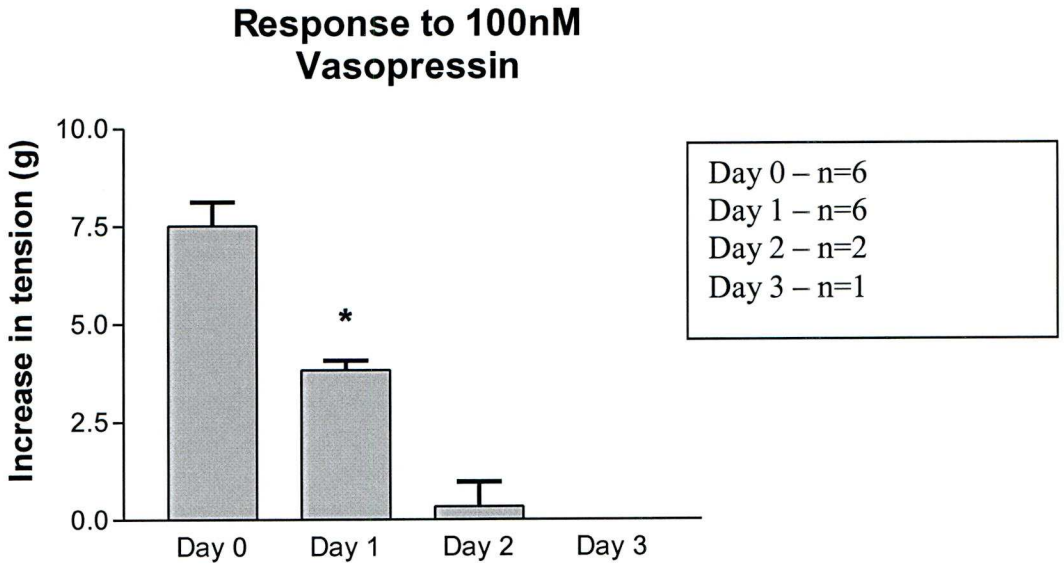

Figure 4.8 - Responses to 100nM vasopressin in human RA rings on consecutive days. Rings were treated with 6mM PhB in theatre. Bars represent mean ±SEM. * Denotes significant decrease in tension from the previous day (p=0.0005)

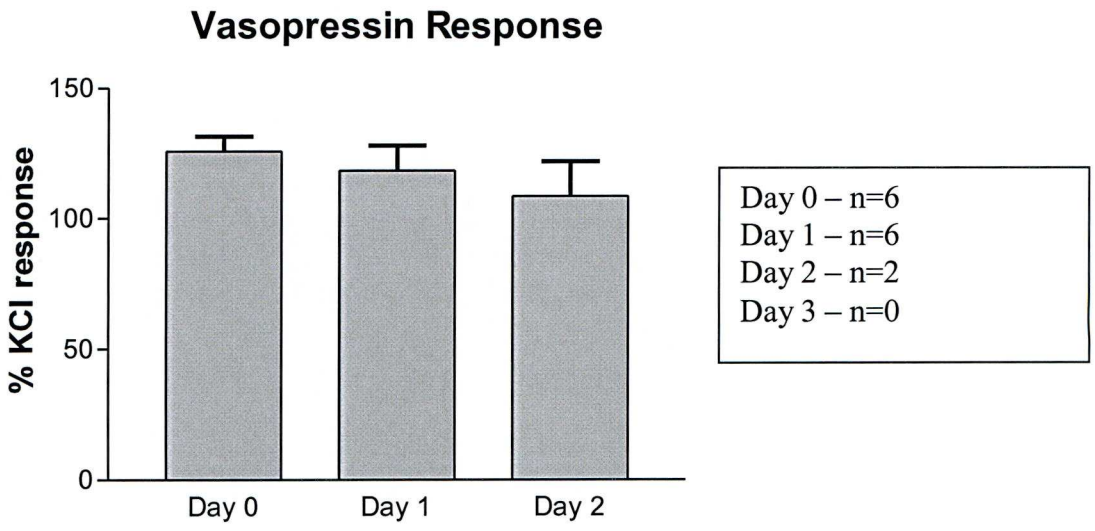

Figure 4.9 – Responses of human RA rings cultured under on consecutive days expressed as % of initial 90mMKCl response of that RA ring. Rings were incubated in DMEM at 37^0C under 6g tension. Bars represent mean ±SEM. There was no significant difference in % 90mM KCl between days

Similar experiments were repeated with 3 rings from each of 4 patients with the rings incubated under different amounts of tension (2 patients (6 rings) 3g, 2 patients (6 rings) 12g) to investigate whether this affected the rings ability to contract beyond day 2. Similar results were seen to those incubated under 6g of tension, with no rings responding on day 3. Due to the small numbers statistical analysis was not possible on this group of experiments.

4.4 Discussion

In human RASMC treated with PhB, the response to NA is fully restored after 48hours. No "receptor escape" owing to up-regulation of newly formed α adrenoceptors was demonstrated. The results were similar in RASMC incubated in the presence of 100nM angiotensin II, 100nM ET-1, 100nM vasopressin and 10μM NA.

Previously it at been shown that there was an increased expression of α adrenoceptors in rat aortic smooth muscle cells that were treated with PhB, when incubated in the presence of angiotensin II (168) Using $[Ca^{2+}]_c$ as a determinant of receptor activation I was unable to demonstrate any increased response of human RASMC to NA when incubated in angiotensin II. This may be a species specific phenomenon although Mussa did not show any increased sensitivity to phenylepherine in his *in vivo* model, however, the levels of angiotensin II would not have been as high in his *in vivo* model as in the cell culture model (147)
In the organ culture model, we were not able to demonstrate any response to NA up to 3 days following treatment with PhB. This was probably due to failure of the RA rings to maintain a contractile response past this time point.

Reviewing the literature no other group appears to have been able to see a regeneration of α-adrenoceptors *in vitro*. Harrison *et al.* maintained human RA rings in an organ bath for 18 hours. At 18 hours rings treated with PhB did not respond to NA, however the contractile force to KCl was similar to the start of the experiment (222). Mussa *et al.* maintained rat aorta rings in an organ bath for 12 hours. At 12 hours rings treated with PhB did not respond to NA, (147) Velez *et al.* concluded that PhB was effective for at least 48 hours in dog radial arteries (221). Their

experiments differed from the ones described here as they did not incubate the radial arteries under tension. In the control group in this paper they showed responses to norepinephrine and phenylephrine remain similar when expressed as a % of the initial KCl contraction after 24 and 48 hours (221). This is similar to what was demonstrated in the experiments in this chapter with vasopressin. They do not report the actual strength of contraction, therefore one is unable to comment whether they saw the same decrease in maximal force of contraction that was demonstrated above. The decrease in force of contraction that was demonstrated is likely to be due to a decrease in the contractile function of the artery rather than a decrease in membrane receptors, because the contractile response to vasopressin was similar at 0, 24 and 48 hours when expressed as a percentage of the KCl response, although the actual force pulled was significantly less.

These studies and the organ culture study carried out in this experiment are limited as *in vitro* studies cannot accurately reflect the clinical situation. To try and mimic more accurately the clinical situation the rings were incubated in DMEM in this experiment which would hopefully provide the nutrients necessary for regeneration of receptors. The rings were also incubated under tension in order to maintain a contractile response. Despite these two measures we were unable to achieve a response past 2 days. It may be that continual contraction followed by relaxation (as happens *in vivo)* is needed to maintain the contractile response.

Mussa *et al.*used an animal model to look at the duration of action of PhB. They found that 50% of the response to phenylephrine had returned at 24 hours (147). This is similar to what we found in our RASMC model.

In human RASMC there is not an up-regulation of the α-adrenoceptors following treatment with PhB and incubation in angiotensin II. This is important clinically because plasma levels of angiotensin II are raised following CABG. Treatment with PhB has no affect on the sensitivity of the human RA to NA..

Chapter 5

Alternative Irreversible Vasodilators

5.1 Introduction

In CABG superior patient survival rates and increased freedom from major adverse cardiac events have been obtained in patients receiving LIMA grafts as opposed to saphenous vein grafts (223). These results have encouraged surgeons towards a complete arterial revascularisation strategy. Promising patency rates obtained with the RA have now also established this graft as a good alternative to the right IMA (85). The RA is a comparatively reactive graft and vasodilator strategies are topically applied by many surgeons in theatre and intravenously in the postoperative period to ameliorate vasospasm, which is believed to account for a proportion of early graft failures (85;86). Spasm can occur both during harvesting and after the graft is anastomosed to the heart, the aetiology is likely to be multifactorial (164). Factors probably involved in the mechanism of spasm include surgical trauma, locally released vasoconstrictors, neural factors, and circulating hormones.

Various different drugs have been used to prevent and treat spasm. Pharmacological strategies to prevent spasm usually consist of an intraoperative strategy and a postoperative strategy. Vasodilator therapies include combinations of nitrates, phosphodiesterase inhibitors or calcium channel antagonists, and the recently introduced irreversible α adrenoceptor antagonist PhB (85;88;224). The beneficial effects of this agent have been shown in a recent prospective study. Patients receiving PhB-treated grafts had a lower level of perioperative myocardial injury and a reduced incidence of adverse cardiac events when compared to a verapamil/glyceryl trinitrate treated group (88). The strategy of using an irreversible inhibitor is very attractive, since it means that pharmacologically effective concentrations can be applied selectively to the graft and the effect maintained in the perioperative period. The benefits of irreversible antagonists in CABG have been

shown as PhB treatment completely abolishes NA-induced contraction in RA for up to 48 hours *in vivo* (221).

Vasoconstrictors other than catecholamines, released into the plasma in the immediate postoperative period can still reduce flow through the RA graft (86). As well as an irreversible α adrenoceptor antagonist, PhB is a weak CaM antagonist (225). However, in previous work we found that PhB had no effect on the contractile response to ET 1, angiotensin II, vasopressin and the depolarisation induced by KCl (92). These results suggest that PhB will only prevent spasm due to stimulation of the α–adrenoceptor. Since the individual contribution of various vasoconstrictors to the generation of the onset of spasm is unknown, we concluded that PhB should not be used as the sole agent in the prevention of RA spasm.

The RA produces stronger vasoconstriction than IMA response to virtually all vasoconstrictors studied (94;169;170;226); however vasoconstriction is equivalent when normalised to account for the different vessel diameters in most cases. However, normalised responses to both angiotensin II and vasopressin in the RA are stronger and more sensitive than in the IMA, and furthermore occur irrespective of the presence of endothelium (94;169;170). In addition, vasopressin-induced contraction in the RA is comparatively resistant to milrinone and glyceryl trinitrate, two of the most commonly used vasodilator strategies (94). The attenuation of the vasopressin-induced contraction in RA grafts would be particularly advantageous for surgeons, since they may wish to use vasopressin to treat hypotension in the postoperative period (104;171).

The aim here is to test whether other irreversible antagonists, with a broader range of activity and the potential to inhibit both receptor and non-receptor-mediated contraction in the RA have the potential for clinical application. We tested two drugs used currently in other clinical settings, fluphenazine mustard and minoxidil sulphate. Fluphenazine mustard is a cell permeable, irreversible antagonist of CaM (172), the cellular protein that translates an increase in intracellular calcium into contraction. It is used clinically as an antipsychotic drug for the treatment of schizophrenia and other psyschoses. Minoxidil sulphate binds irreversibly to the vascular ATP-sensitive K^+ channel (K_{ATP} channel) and is used as an antihypertensive agent (174). It is used clinically to treat severe hypertension resistant to other drugs and hair loss. Since to be able to attenuate vasopressin induced contraction would have clinical relevance, I examined the ability of fluphenazine mustard and minoxidil to inhibit vasopressin induced contraction. Finally, the rho-kinase pathway will be investigated as another possible common pathway that could be blocked to prevent spasm if an irreversible agonist becomes available.

5.2 Methods and Materials

Samples of RA were obtained from patients undergoing CABG. They were harvested as described in chapter 2. RA rings were set up and pre-tensioned in the organ bath as described in chapter 2. 108 rings were used in total.

To test for functional contractility, arterial rings were first stimulated with 90 mM KCl and all other responses, including later KCl responses, expressed as % of this response. Endothelial function, as evidenced by a vasodilatory response to 10 mM acetylcholine in rings pre-contracted with 30 mM KCl, was found to be negligible. Responses were terminated by washing with three complete changes of media. Rings pretreated with 100μM fluphenazine-N-2- chloroethane, diHCl (SKF7171A, HCl;

Merck Chemicals Ltd., Nottingham, UK) were incubated in the presence of the drug for 40 minutes. Rings treated with minoxidil sulphate (Merck Chemicals Ltd.) were incubated for 30 min at 10 mM, a concentration reported to completely reverse NA-induced contraction in vascular smooth muscle (227). Following incubation with fluphenazine or minoxidil sulphate the drug was removed by three complete changes of media. Responses to angiotensin II (Sigma, Poole, UK) were recorded within 20 min of drug washout and responses to Arg-vasopressin (Sigma) at either 30 or 120 min.

Responses in treated rings.were normalised to control rings stimulated in parallel and pretreated with vehicle alone (dimethylsulphoxide; DMSO). Y27632 (Merck Chemicals Ltd.) or glyceryl trinitrate (DBL, Warwick, UK) which were added to the organ chamber 5 min prior to and during agonist addition.

5.2.1 Data analysis

Data is presented as mean ± standard deviation of the mean (SD), where n refers to the number of independent samples tested. The threshold vasopressin concentration was defined as the concentration at which tension was significantly raised above baseline tension. Data were tested for normal distribution and appropriate comparisons were undertaken as the analysis dictated using a one-way ANOVA and a Bonferroni correction for multiple comparisons. All analyses were carried out using the program Arcus QuickStat Biomedical using a p value of 0.05 (Hearne Scientific Software, Dublin, Eire).

5.3 Results

RA samples were collected from 31 patients. 108 rings were used in total. The clinical characteristics of these patients are given in table 1.

Total number of Patients	31
Mean age (years)	63
Sex ratio (Male:Female)	23:8
Risk Factors (n)	
Smoking	6
Hypertension	15
Diabetes mellitus	6
Preoperative Medication (n)	
β–blockers	22
Calcium channel blockers	14
ACE inhibitors	11
Nitrates	15
Potassium channel openers	9

Table 5.1 - Patient Characteristics

The internal diameter at a pressure of 100 mmHg was 3.8 ± 0.9 mm. To test for functional vasoconstriction, sections of human RA were stimulated with 90 mM KCl that raised tension from a mean basal value of 1.93 ±0.92 g (n = 31) to 7.70 ± 3.66 g. Data was collected from distal arterial sections treated with PhB in theatre (6.26 ± 2.18 g; n = 17) and proximal sections treated with papaverine (9.72 ± 4.49 g; n = 14). As previously reported by other investigators (228), proximal sections gave significantly higher KCl-induced contraction than distal ($p < 0.05$). All data were therefore expressed as % of this initial KCl response.

5.3.1 Vasoconstrictor Responses in the Radial Artery

Robust concentration dependent contraction was observed to vasopressin and angiotensin II giving average maximal responses of 109±16% KCl (n=13;95% confidence interval 104-115) and 95.4±6% KCl (n=14; 95% confidence interval 79-104) respectively. EC_{50} values (95% confidence intervals) of 0.51nM (0.27-0.98) and 3.54nM (1.73-7.24) were obtained for vasopressin and angiotensin II respectively. Proximal samples gave significantly higher maximal responses to angiotensin II and vasopressin ($p<0.05$), but when normalized to KCl there was no significant difference between proximal and distal samples ($p=0.44$). Representative traces are shown in Figure 5.1a&b.

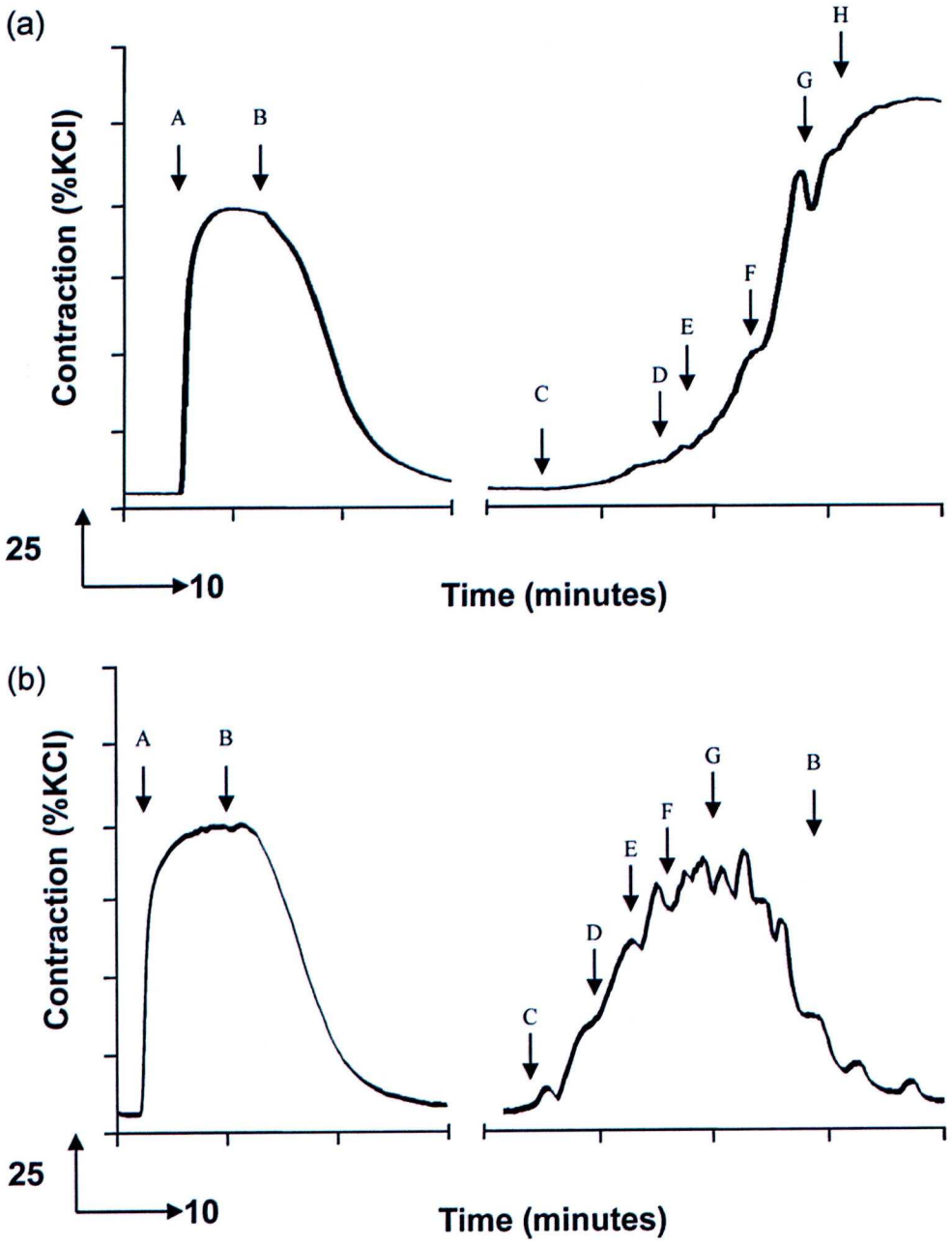

Figure 5.1 - Representative traces from individual rings of RA showing standard responses to increasing concentrations of either vasopressin or angiotensin II. Responses are shown in relation to an initial response to 90 mM KCl (*A*). Following washout (*B*) of the KCl response with two complete changes of media, rings were stimulated with increasing concentrations of either vasopressin (a) where *C* = 0.1, *D* = 0.3, *E* = 1, *F* = 10, *G* = 30 and *H* = 100 nM vasopressin) or angiotensin II (b) where *C* = 1, *D* = 3, *E* = 10, *F* = 30 and *G* = 100 nM angiotensin II).

Whilst maximal responses to both KCl and angiotensin II returned to baseline following agonist washout within 10 min, vasopressin-induced contraction was significantly reversed only by glyceryl trinitrate concentrations greater than 2μM. An EC_{50} value for the reversal of maximal vasopressin-induced contraction with glyceryl trinitrate was 89.12μM (n=6).

5.3.2 Effects of treatments against vasopressin induced contraction

Pre-treatment with fluphenazine consistently reduced the maximal vasopressin-induced contraction (Figure 5.2a) and significantly increased the EC_{50} (Table 5.2) whereas arterial rings pretreated with minoxidil failed to show any significant effects. Since vasopressin-induced contraction was not reversed by agonist washout, the effects of the Rho kinase inhibitor Y27632 were also tested against vasopressin-induced contraction and compared with glyceryl trinitrate (Figure 5.2b). Treatment of arterial rings with 10μM Y27632 reduced the basal tension by 33±7% (n=5) and significantly reduced the maximal vasopressin-induced contraction (Figure 5.2) and significantly decreased the affinity of the response (Table 5.2). Fluphenazine mustard and Y27632 increased the threshold concentration which significantly raised tension above basal values from 0.3nM in controls to 1.0nM in treated samples from both groups.

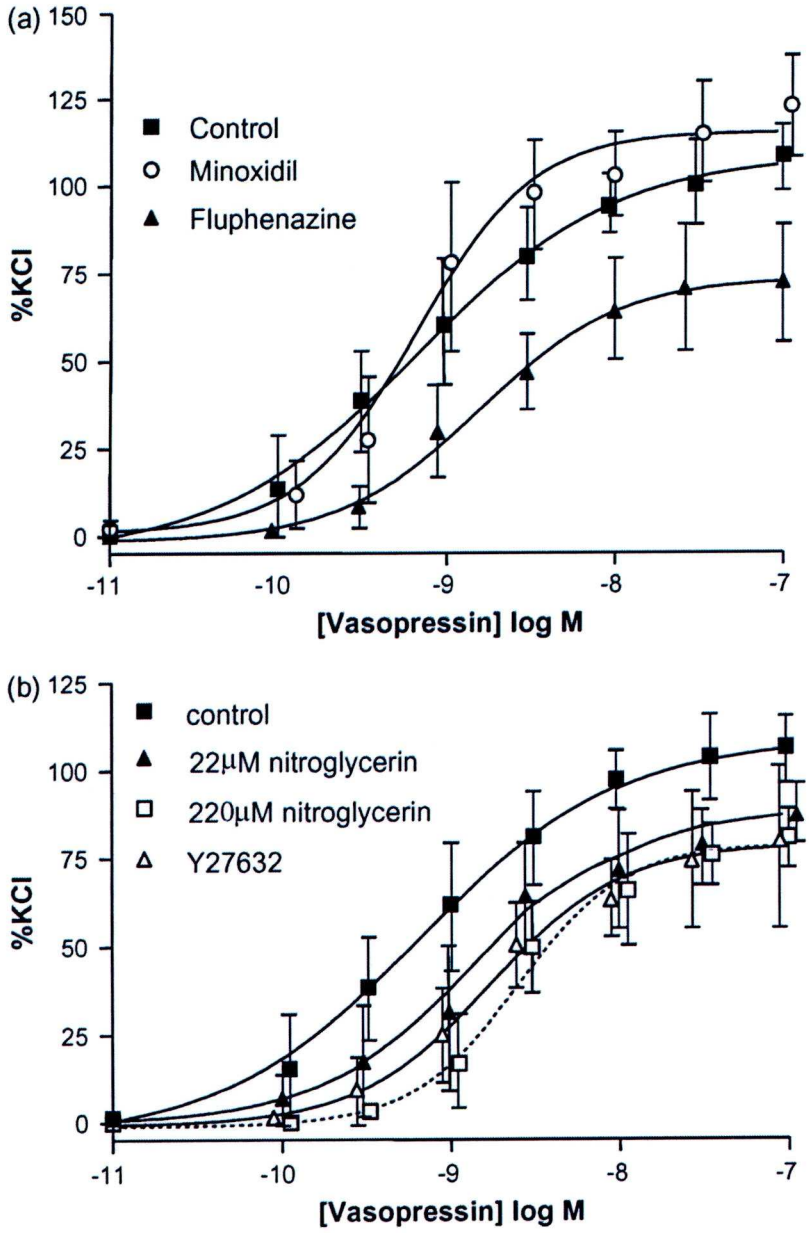

Figure 5.2 - Effect of treatments against vasopressin-mediated vasoconstriction. Cumulative concentration response curves were obtained to vasopressin following pretreatment (a) with vehicle alone (control; *n* = 12), 100 μM fluphenazine mustard (*n* = 6) or 10 μM minoxidil sulphate (*n* = 6) or in the presence of (b) 10 μM Y27632 (*n* = 5) and 22 μM (*n* = 5) and 220 μM (*n* = 5) glyceryl trinitrate, where *n* refers to the number of independent samples tested. Average responses ± SD, are shown relative to a contractile response to 90 mM KCl applied prior to treatment. See table 5.2 for statistical comparisons

Vasopressin	Control	Minoxidil	Fluphenazine mustard	Y27632	Glyeryl trinitrate (22μM)	Glyeryl trinitrate (220μM)
N	13	7	7	5	5	5
Max Response (%KCl)	109±16	119±16	*72±12	*94±13	99±12	81±11
Confidence Intervals (95%)	104-115	134-105	81-63	110-73	114-83	95-68
EC_{50} (nM)	0.51	0.74	*1.26	*1.54	1.65	*2.75
Confidence Intervals (95%) (nM)	0.27-0.98	0.37-1.45	0.60-2.63	0.60-3.89	0.49-5.62	0.93-8.13

Table 5.2 - Data is expressed as % response to 90mM KCl. *Denotes significant differences ($p < 0.05$) between treatment groups and controls by ANOVA using the Bonferroni correction for multiple comparisons

5.3.3 Effect of minoxidil sulphate and fluphenazine mustard pretreatment against angiotensin II and KCl-induced vasoconstriction

Maximal responses to angiotensin II were significantly reduced by pre-treatment with fluphenazine mustard but not minoxidil when compared to controls (Table 5.3). No measurable change in EC_{50} values for angiotensin was observed with either treatment. In addition fluphenazine mustard significantly reduced contraction elicited by 90mM KCl by 48±15% when compared to control response. In control rings, KCl responses were maintained, giving average values 107 ± 21% of the initial contraction (n = 8). Pretreatment with minoxidil sulphate showed no measurable

effect against KCl-induced contraction giving an average response 112 ± 17% (n = 6) of the initial contraction

Angiotensin II	Control	Minoxidil	Fluphenazine
N	14	7	6
Max Response (%KCl)	95.4±6.4	80±28	*54±20
Confidence Intervals (95%)	79-104	107-55	75-32
EC_{50} (nM)	3.54	5.24	7.41
Confidence Intervals (95%) (nM)	1.73-7.24	2.04-13.80	0.3-173nM

Table 5.3 - Comparison of maximal responses and affinity of response to angiotensin II in human RA pretreated with minoxidil sulphate or fluphenazine mustard. Data is expressed as % response to 90 mM KCl. *Denotes significant differences (p < 0.05) between treatment groups and controls by ANOVA using the Bonferroni correction for multiple comparisons

5.4 Discussion

The major finding of this work was that pre-treatment of RA grafts with the irreversible CaM inhibitor, fluphenazine mustard (SKF7171A), significantly attenuated receptor-mediated contraction evoked by angiotensin II and vasopressin, and depolarisation-induced contraction by high potassium. CaM is the cellular protein, which integrates the calcium response and activates the myosin light chain kinase responsible for contraction. Since an increase in smooth muscle calcium is the primary initiating stimulus for contraction, this makes CaM an excellent target for inhibiting contraction evoked by a wide range of agonists. This study also demonstrated that the reversible rho kinase antagonist, Y27632 reduced the maximal vasopressin-induced contraction.

We chose to test the actions of the inhibitors against vasopressin and angiotensin II because normalised responses to both angiotensin II and vasopressin in the RA are stronger and more sensitive than in the IMA, and occur irrespective of the presence of endothelium (94;169;170) therefore it is important to be able to attenuate these responses. In addition, vasopressin-induced contraction in the RA is comparatively resistant to milrinone and glyceryl trinitrate, two of the most commonly used vasodilator strategies (94). The inhibition of the vasopressin-induced contraction in RA grafts would be particularly advantageous for surgeons, since they may wish to use vasopressin to treat hypotension in the postoperative period (104;171).

Vasopressin has been shown to be a potent vasoconstrictor in the IMA (101) with calcium channel antagonists causing only limited relaxation and GTN causing nearly full relaxation but failing to prevent contraction evoked by vasopressin. Contractions

could be fully blocked by the V1 receptor antagonist 1-deaminopenicillamine, 4-valine, 8-D-AVP (101).

We have previously shown that the only currently used irreversible antagonist, PhB, has no effect on preventing angiotensin II and vasopressin induced contraction of the RA. As spasm is likely to be multi-factorial, blockage of these vasoconstrictors may be clinically important.

5.4.1 Calmodulin inhibitors

Fluphenazine mustard (SKF7171A) significantly attenuated receptor-mediated contraction evoked by angiotensin II and vasopressin, and depolarisation-induced contraction by high potassium concentrations.

Although effects were only measured up to 2 h after treatment they would be expected to be similar in duration to PhB, since irreversible interactions of this type will last until the smooth muscle cells of the vessel wall replace the inactivated protein . Inhibition with fluphenazine mustard should therefore last well into the postoperative period (147); however it would be useful to verify this in vivo. The animal model used to confirm the duration of action of PhB would suit this purpose (147). Using this model it was demonstrated that the effects of PhB lasted for 16 h *in vivo*.

As well as being involved in contraction of vascular smooth muscle, CaM is involved in a number of cellular functions that cause vasodilatation. Nitric oxide is synthesised by vascular endotheliun by a family of CaM-dependent NO synthases.(NOS). Caveolin, the principle structural protein in calveolae, interacts

with endothelial NOS (eNOS) to inhibit it in a reversible process modulated by CaM. CaM also increases Ca^{2+} release from sarcoplasmic reticulum and reduces Ca^{2+} uptake. Fluphenazine may also therefore cause vasodilatation by inhibiting these processes.

5.4.2 Potassium Channel Openers

We found 10mM minoxidil sulphate to be ineffective at inhibiting contractions in the RA. This occurred irrespective of the use of potassium channel blockers in the preoperative medication. Interestingly, minoxidil sulphate demonstrates a high degree of tissue and species selectivity. For example, minoxidil sulphate is a potent vasodilator in rat aorta but not in rabbit aorta or rat mesenteric artery, whereas other K_{ATP} channel openers were equally effective in all tissues (227). The lack of effect measured in human RA in this study indicates that minoxidil sulphate has poor activity in this vessel also. It is unlikely that the lack of effect is due to the concentration used, as we chose to use a concentration more than ten times that reported as maximal.

5.4.3 Rho Kinase Inhibitors

The effect of the reversible Rho kinase inhibitor Y27632 was also investigated. The sustained phase of vascular smooth muscle contraction is thought to involve Ca^{2+} sensitization (179). It is believed to be this phase that initiates clinical vasospasm in cerebral arteries (180). The major mechanism of the Ca^{2+} sensitization of contraction is through the inhibition of the smooth muscle myosin light chain phosphatase, resulting in increased myosin light chain phosphorylation and smooth muscle contraction at a constant intracellular calcium level (179). It has been recognised that the monomeric G protein Rho and its downstream target Rho-kinase can

participate in sustained vasoconstriction by phosphorylating and inhibiting myosin binding (181). Rho-kinase has been proposed to play a variety of vascular smooth muscle disorders including hypertension, coronary and cerebral vasospasm (180;182;183).

Rho kinase is activated by 5-HT, ET 1 and thromboxane A2 [17], NA (229) and by the stress hormone cortisol (230). This study demonstrated that Y27632 reduced the maximal vasopressin-induced contraction Therefore the involvement of the Rho kinase pathway in vasopressin induced contraction in the RA indicates that both sensitivity of the graft to vasopressin. This may be pertinent to the management of postoperative contraction in RA grafts or the use of vasopressin in the management of hypotension following CABG (171), particularly as vasopressin-induced contraction in RA is refractory to two of the most commonly used vasodilator strategies (94).

The mRNA expression of rho-kinase is enhanced in inflammatory and atherosclerotic lesions causing hypercontraction of the artery. In cultured human coronary artery smooth muscle cells the expression of rho kinase is enhanced by angiotensin II and interleukin 1β. This could be an important contribution to vasospasm following CABG as both levels of angiotensin II and interleukin 1β are raised (91). .

As well as its role in vascular smooth muscle contraction rho-kinase plays a role in mediating various other cellular functions. These include actin cytoskeleton organisation (231), cell adhesion and motility (232), cytokenesis (233) and gene

expression (234). All of these may be involved in the pathogenesis of atherosclerosis. Furthermore, rho-kinase is involved in endothelial contraction that increases endothelial permeability and hence enhances atherosclerosis (235). In porcine femoral arteries balloon injury-induced neointimal formation was significantly reduced by Y27632 (236). As well as preventing spasm, the long-term use of rho-kinase inhibitors may also suppress the development of atherosclerosis.

The rho-kinase inhibitors fasudil and Y 27632 act in a competitive manner with ATP (237). They decrease the myosin light chain phosphatase inhibition and subsequent elevation of myosin light chain phosphorylation, mediated by receptor stimulation or the activation of G-proteins. They have no effect on the transient or sustained increase in intracellular calcium (238;239). Y2632 was as effective as high dose GTN at preventing vasopressin induced contraction of the RA in our study. These large concentrations of GTN could not be achieved systemically as they would cause profound hypotension. Fasudil has been shown to be able to prevent vasospasm at clinically useful concentrations (240). Also there is the problem with tolerance to long-term GTN. This has not been shown to be a problem with rho-kinase inhibitors.

The drop in basal tension seen in the RA on addition of Y27632 has been described in the mammary artery by Batchelor *et al.* (158). Rho A appears to be basally active (241), therefore it follows that Y27632 will inhibit resting tone. This does not seem to be the case in the coronary arteries in vivo, as treatment with fasudil had no effect on nonspastic segments of artery following acetylcholine induced vasospasm (240). Also it has been shown that rho-kinase is involved in the increased systemic vascular

resistance in hypertensive patients but not normotensive controls (240). It remains to be seen whether the inhibition of the basal tone of the RA and IMA occurs ex-vitro.

We chose to pretreat the RA with rho-kinase inhibitors rather than apply a vasoconstrictor and reverse any spasm because we feel prevention of spasm is the goal. It has previously been suggested that pretreatment with Y27632 is less effective than application following the vasoconstrictor agonist (241;242). This discrepancy does not appear to be a major factor in human conduit artery (158). It also does not appear to be a factor in vivo in human coronary arteries (240).

Y27632 was as effective at preventing contraction of the RA as high concentrations of GTN. This is not suprising as nitric oxide induces vasodilatation of vascular smooth muscle by a cGMP dependent kinase (243). Since NO dilatations occur without changes in intracellular calcium concentration, calcium sensitisation must be involved. Myosin light chain phosphatase is activated by NO, stimulation of the rho A/rho kinase pathway antagonises the effects of NO that, in turn, can be restored by Y27632 suggesting a role of the rho kinase pathway in NO. The RA undergoes a period of hypoxia prior to grafting. Hypoxia has been shown to cause vasoconstriction in the pulmonary artery or saphenous vein (244). This may in part be due to a decreased expression of endothelial nitric oxide synthetase (eNOS). This reduction in eNOS expression can be prevented by rho-kinase inhibitors (244). Thus rho-kinase inhibitors may be beneficial in RA storage solutions prior to grafting, not only to cause vasodilataion directly through increasing myosin light chain phosphatase activity, but also but by preventing any decrease in eNOS expression induced by hypoxia.

In conclusion the rho-kinase inhibitor Y27632 was as effective as high dose GTN at preventing vasopressin induced contraction of the human RA *in vitro*. Although Y27632 is not clinically available, fasudil, another rho-kinase inhibitor is available in intravenous and oral preparations. As well as using long term administration of rho-kinase inhibitors, the development of an irreversible rho-kinase inhibitor would allow a once off treatment in theatre prior to grafting to prevent spasm.

5.4.5 Conclusions

Fluphenazine mustard has potential to be developed as a useful treatment in the prevention of RA spasm and demonstrates long-lasting effects against vasoconstriction induced by several unrelated agonists. Further studies are required to investigate the duration and breadth of effect of fluphenazine mustard against a broader range of vasoconstrictors in RA. The development of clinically useful rho-kinase inhibitors may also provide a future therapeutic opportunity for the management of arterial contraction in the postoperative period, especially if rho-kinase inhibitors can be developed to give long-lasting protection (246).

Chapter 6

Conclusions

6.1 Summary of key findings

The results of the experimental work in this thesis have shown that there are a large number of nerve endings in the wall of the human RA. Stimulation of RA rings with tyramine caused a contraction that is blocked by the irreversible α-adrenoceptor agonist, PhB. After relaxation with GTN, further addition of tyramine did not cause a contraction suggesting depletion of the nerve endings in the wall of the RA of NA. Stimulation of RASMC by tyramine did not cause an increase in peak calcium fluorescence, but direct stimulation with NA did. It was therefore concluded that nerve endings in the wall of the RA contain significant amounts of NA and may therefore cause spasm as they degenerate. Treatment of RA rings with reserpine decreased the strength of contraction to tyramine. This may be a way of degranulating nerve endings clinically, thus reducing the problem of spasm.

In human RASMC there is not an over-expression of α-adrenoceptors after treatment with PhB, even when cultured in the presence of other vasoconstrictors including angiotensin II.

The irreversible CaM inhibitor fluphenazine mustard significantly lowered the maximal contractile response in human RA rings to vasopressin and angiotensin II, and significantly lowered the EC_{50} in human RA rings to vasopressin. The irreversible KCO, minoxidil sulphate had no effect on the maximal contractile response and EC_{50} in human RA rings to vasopressin and angiotensin II. Fluphenazine mustard may therefore be used to prevent spasm in the RA due to a number of different vasoconstrictors.

The rho-kinase inhibitor, Y27632 significantly lowered the maximal response and the EC_{50} in human RA rings to vasopressin. The rho-kinase system could be blocked in the future, if an irreversible rho-kinase inhibitor becomes available to prevent spasm in the RA.

6.2 General Conclusions

It is now widely accept that the IMA should be used as a conduit to the LAD. The use of more than one arterial graft has not generally been accepted. Only 15% of patients undergoing CABG in the UK have more than one arterial graft (26). There is strong evidence in the literature to support the use of BIMA in terms of survival and fewer ischemic cardiac events (17-19). The main reason for the lack of use of BIMA is probably the increased incidence of sternal wound infections and the greater complexity of the operation. Use of the RA does not increase the complexity of CABG and is associated with a lower incidence of conduit harvest wound site infection. The reasons for lack of widespread use of the RA are probably two fold; firstly the lack of large randomised control trial showing an increased patency rate of the RA over the long saphenous vein, and secondly the fear of spasm of the RA.

Although excellent short and mid-term patency rates of the RA have been demonstrated, only Possati has published long-term results (44). This show superior patency rates to historical patency rates of the saphenous vein. The patency rates of the saphenous vein have improved over the last 10 years due to changes in surgical technique and the routine use of statins and asprin postoperatively, and therefore it is unfair to compare the patency rates of the RA to that of the saphenous vein 10 years ago. There is little scientific evidence comparing the RA and the SV. Trials comparing the two conduits are in progress and the interim results of one of these

trials has showed no difference in patency at 5 years or a difference in clinical events (46). Long-term results are awaited, as this is when the patency rates of the RA is expected to be greater.

The true incidence of spasm of the RA is unknown and difficult to properly assess. The incidence of spasm quoted in the literature is between 0 and 10% (34;40;48;49;56-58). These figures are based on elective angiography during the first year following surgery. The true incidence of spasm is therefore probably higher than this as the RA is believed to be more prone to spasm in the early postoperative period, when native coronary flow is still present, and hence may go undetected.

The use of the irreversible α adrenergic blocker, PhB provides an attractive new way to treat spasm in the post-operative period (143). However, it makes one major assumption, that spasm is due to α-adrenergic agonists. It is not known what contribution circulating catecholamines make to spasm. Levels of circulating NA is raised by 3-4 times for the first 24 hours following cardiac surgery (91). These levels are 10 times less than that required to cause contraction of RASMC or RA rings *in-vitro*. NA is released from sympathetic nerve terminals, local concentrations of NA will therefore be higher than systemic concentrations, and may be at sufficient levels to cause spasm. We have shown that sympathetic nerve endings in the wall of the RA have the potential to degranulate and cause a contraction similar in strength to the maximal response to NA. Degenerating nerve endings in the wall of the artery provides an attractive hypothesis for the mechanisms of spasm in the postoperative period and could explain why spasm can occur many weeks following surgery when one would have expected the effects of surgical trauma to the artery to have resolved

and circulating levels of catecholamines and other vasoconstrictors to have returned to normal.

If degenerating nerve endings are a cause of spasm, PhB would provide an attractive way of preventing spasm in the initial postoperative period. We have shown that the effects of PhB last for up to 48 hours. We were unable to demonstrate an overshoot in α adrenoceptors following PhB treatment of human RASMC, suggesting there will not be an increased propensity of spasm of the RA following PhB treatment.

After 48 hours the effects of PhB are no longer present and therefore one has to look at other methods to prevent spasm. Calcium channel blockers and nitrates are the most commonly used drugs but there is little evidence to show they are of any benefit (44;45). Nerve endings may be degranulated at the time of surgery to prevent them releasing NA. Tyramine degranulates nerve endings, however the nerve endings are probably still capable of refilling their vesicles by reuptaking NA or producing it. Reserpine inhibits the amine uptake process in the vesicle membrane and thereby allows leakage of NA into the cytoplasm of the nerve cell where it is broken down by neuronal MAO. The effects of reserpine should last at least 10 days because recovery of neuronal function depends on synthesis of new vesicles and their transport to the axon terminals, the effects may however be permanent if the nerves are no longer functional. We have demonstrated that reserpine prevents an increase in tension of RA rings in response to the addition of tyramine.

Although we believe degenerating nerve endings to be one of the major contributors to spasm of the RA, the aetiology is probably multi-factorial, and therefore any treatment strategies should take this into account. Surgical techniques have

improved over the last few decades, which probably led to the revival of the RA. Various vasoconstrictors have been implicated in the cause of spasm and antagonists to these vasoconstrictors could be used to treat spasm. This would mean using a cocktail of drugs, many of which are not yet available clinically and most of which are reversible. A more feasible strategy is to block the contractile mechanism further downstream where inhibition of one enzyme will prevent vasoconstriction due to a number of stimuli. This is the principle for the use of calcium channel blockers and phosphodiesterase inhibitors. Other drugs that fit into this category are CaM inhibitors, KCO or rho-kinase inhibitors. The experimental work in this thesis has shown that the irreversible CaM antagonist fluphenazine mustard is effective at decreasing contractions of human RA rings due to vasopressin and angiotensin II. Minoxidil, an irreversible KCO was ineffective. It is therefore unlike that it has any role in the prevention or treatment of spasm.

Fluphenazine may therefore have role as an antispasmodic. Fluphenazine acts in a completely different way to other commonly used antispasmodics such as GTN and phosphodiesterase inhibitors, so its effects may be additive to any of these other agents, if used in combination with them.

The rho-kinase pathway is another pathway that may be blocked to have an effect on a wide range of agonists. The rho-kinase inhibitor Y27632 was effective in increasing the EC_{50} and decreasing the maximum contraction to vasopressin and angiotensin II. Although Y27632 is not available clinically, fasudil, another rho-kinase inhibitor, is available in an oral and intravenous preparation and could be used in the intra-operative and postoperative period. As yet rho-kinase inhibitors are not

irreversible. An irreversible rho-kinase inhibitor would be a very attractive drug in the prevention of spasm of the RA.

The use of the RA has increased over the past five years. The attractions of the RA to the surgeon are immediate and obvious; it is a versatile conduit that can be harvested easily and safely, it has handling characteristics superior to other arterial grafts, and it reaches comfortably any target vessel (247). It has been shown to have excellent mid and long-term patency rates (42;45;48;49;57;248-250). The superiority over the long saphenous vein has yet to be shown, however midterm results are similar and on the basis of long term patency rates of RA grafts and with the previous experience of persisting patency in arterial grafts and occlusion of vein grafts the continued use of the RA is justified. With better understanding of the mechanisms of spasm and new drugs becoming available to treat it, spasm will not be the problem feared by some.

Appendix

pH of phenoxybenzamine in storage solutions

Taggart initially described using 6mM PhB (193). This was the dose used in theatre at The Cardiothoracic Centre, Liverpool at the time the experimental work for this thesis was carried out. In the experiments described in Chapter 4, problems with the cells lifting from the bottom of the 96 well plates was encountered. One of the reasons for this was thought to be the acidic nature of PhB. This was therefore investigated. As clinical practice differs from these *in-vitro* experiments, because the PhB is placed in blood, the effect on blood pH was also investigated. The experiments were repeated at room temperature and 37°C.

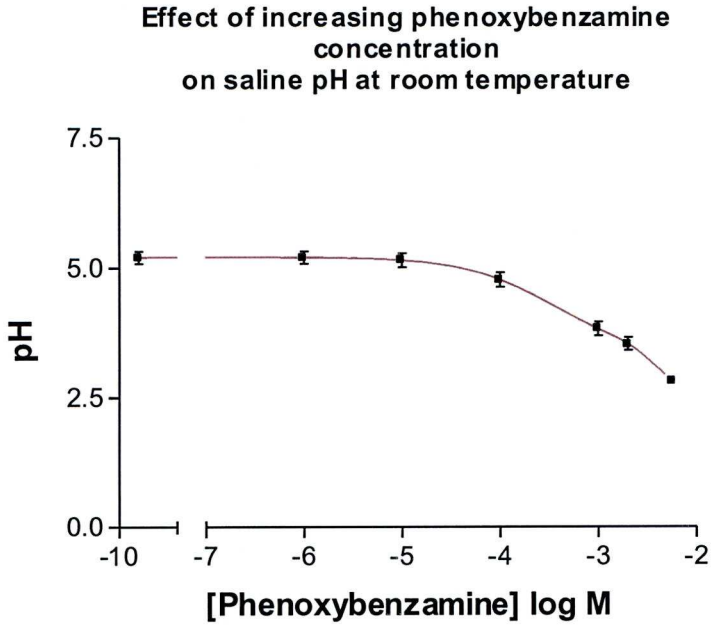

Figure A1 – Effect of increasing phenoxybenzamine concentrations on saline pH at room temperature (n=3). Mean responses ±SEM are shown.

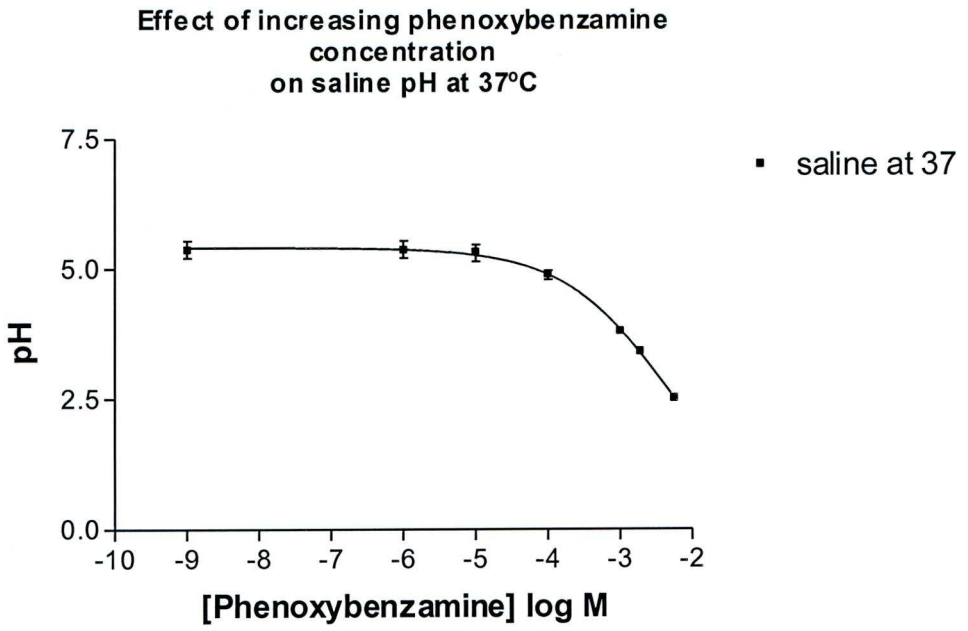

Figure A2 – Effect of increasing phenoxybenzamine concentrations on saline pH at 37°C. (n=3). Mean responses ±SEM are shown.

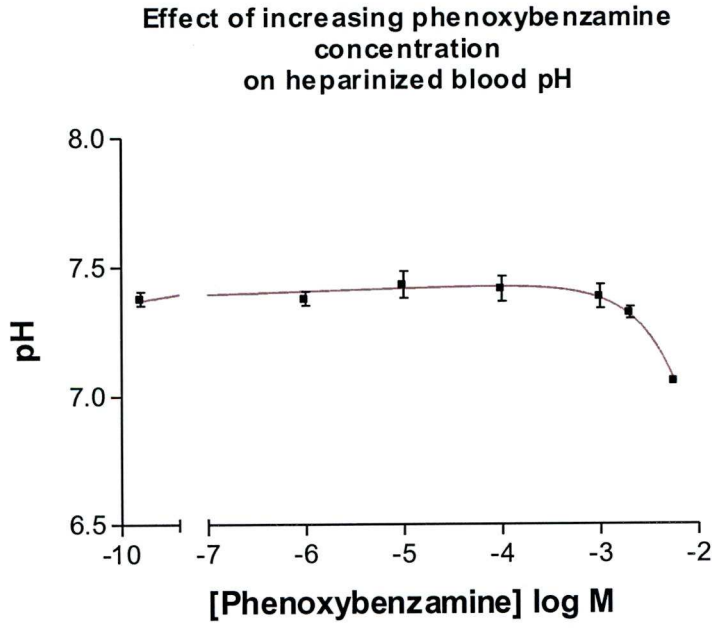

Figure A3 – Effect of increasing phenoxybenzamine concentrations on heparinised blood (100iu/ml) pH at 37°C (n=3). Mean responses ±SEM are shown.

It had been shown by others that a concentration of 1μM PhB completely blocks the contractile response to NA (221). Due to this and the findings described above the surgeons at The Cardiothoracic Centre, Liverpool, now use a lower concentration of PhB

Potentiation of noradrenaline response by vasopressin in RASMC

As mentioned in this thesis previously the cause of spasm is unknown. The levels of vasoconstrictors in the plasma post-operatively are insufficiently raised to cause a contraction of the RA *in vitro* alone, it has been shown that there is a synergistic effect between some vasoconstrictors e.g. NA and vasopressin. It may therefore be a combination of vasoconstrictors that are the cause of spasm *in vivo*.

Various concentrations of NA was added to RASMC on 96 well plates containing various concentrations of vasopressin. The peak increase in fluorescence was measured (see figure A5).

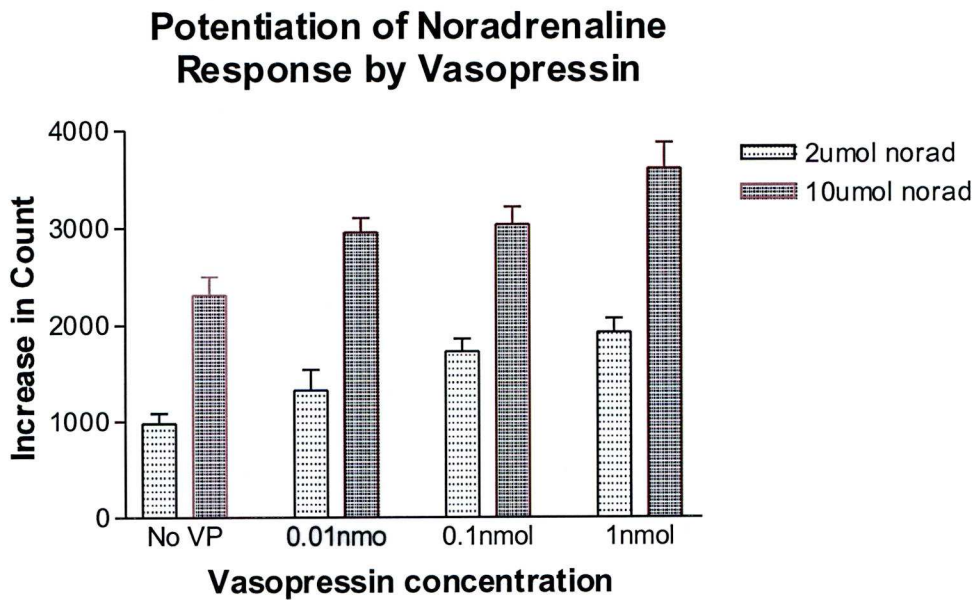

Figure A5 – Peak increase in fluorescence of RASMC on addition of 2 or 10μM NA in the presence of varying concentrations of NA. n=18 wells from 3 batches. Bars show mean of 5 readings for each well. Error bars represent SEM.

Detailed anatomy of the Radial artery

The forearm and hand receives its blood supply from the radial and ulnar arteries. The RA appears to be a direct continuation of the brachial artery, but it is smaller in calibre than the ulnar artery. It commences at the bifurcation of the brachial artery, just below the elbow crease. It passes along the radial side of the forearm to the wrist where it winds backward, around the lateral side of the carpus, beneath the tendons of the Abductor pollicis longus and Extensor pollicis longus and brevis. Finally it passes forward between the two heads of the first interosseous muscles, into the palm. In the palm it crosses the metacarpal bones and at the ulnar side of the hand it joins with the deep palmar branch of the ulnar artery to form the deep palmar arch.

Branches of the RA in the forearm include;(1) the radial recurrent artery which arises immediately below the elbow, supplies muscles around the elbow-joint, and anastomosing with the terminal part of the profunda brachi artery. (2) Muscular branches which supply muscles on the radial side of the forearm. (3) The palmar carpal artery is a small vessel which runs across the front of the carpus and anastomoses with the palmar carpal branch of the ulnar artery. This anastomosis is joined by a branch from the palmar interosseous above, and by recurrent branches from the deep palmar arch below, thus forming a palmar carpal net-work which supplies the articulations of the wrist and carpus. (4) Superficial palmar branch which arises from the RA, just where the RA is about to wind around the lateral side of the wrist. It anastomoses with the terminal portion of the ulnar artery, completing the superficial palmar arch.

At the wrist the RA gives off four branches the dorsal carpal artery and the first dorsal metacarpal artery. The dorsal carpal branch is a small vessel which arises beneath the Extensor tendons of the thumb; crossing the carpus transversely toward the medial border of the hand, it anastomoses with the dorsal carpal branch of the ulnar and with the palmar and dorsal interosseous arteries to form a dorsal carpal network. From this network are given off three slender dorsal metacarpal arteries. Near their origins they anastomose with the deep palmar arch by the superior perforating arteries, and near their points of bifurcation with the common palmar digital vessels of the superficial palmar arch by the inferior perforating arteries. The first dorsal metacarpal arises just before the RA passes between the two heads of the first Interosseous dorsalis and divides almost immediately into two branches which supply the adjacent sides of the thumb and index finger; the radial side of the thumb receives a branch directly from the RA

In the hand the RA gives rise to the arteria princeps pollicis. It arises from the RA just as it turns medial towards the deep part of the hand; it descends between the first interosseous dorsalis and adductor pollicis obliquus, along the ulnar side of the metacarpal bone of the thumb to the base of the first phalanx, where it lies beneath the tendon of the flexor pollicis longus and divides into two branches. These make their appearance between the medial and lateral insertions of the adductor pollicis obliquus, and run along the sides of the thumb, forming on the palmar surface of the last phalanx an arch, from which branches are distributed to the integument and subcutaneous tissue of the thumb. The arteria palmaris indicis radialis arises close to the arteria princeps pollicis, descends between the first interosseus dorsalis and adductor pollicis transversus, and runs along the radial side of the index finger to its

extremity, where it anastomoses with the proper digital artery, supplying the ulnar side of the finger.

The deep palmar arch is formed by the anastomosis of the terminal part of the RA with the deep volar branch of the ulnar artery. It lies upon the extremities of the metacarpal bones and on the interossei muscle, being covered by the adductor pollicis obliquus, the flexor tendons of the fingers, and the lumbricales.

The superficial palmar arch is formed by the ulnar artery, and is usually completed by a branch from the volaris indicis radialis artery, but sometimes by the superficial palmar arteryor by a branch from the a. princeps pollicis artery. The arch passes across the palm, describing a curve, with its convexity downward.

RESEARCH INTO VASCULAR DISEASE AND BYPASS GRAFT FAILURE AT THE CARDIOTHORACIC CENTRE

PATIENT INFORMATION

You are being invited to assist in research studies being performed at the Cardiothoracic Centre, in collaboration with the University of Liverpool. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Purpose of the studies

Coronary artery disease is more widespread in the North West than nearly any other region in the World. In this condition fatty blockages obstruct the coronary arteries. Coronary bypass surgery has become a routine operation and over 4000 operations are performed annually in the North West region. The operation involves re-routing blood around the obstruction using a blood vessel (graft) taken from elsewhere in the body. Although the operation is highly beneficial for most patients, chest pain (known as angina) frequently recurs, affecting 50% of patients by 10 years. This pain recurs as a consequence of the grafts themselves becoming blocked. In collaboration with the Department of Human Anatomy and Cell Biology at the University of Liverpool, we have initiated a programme of research aimed at understanding and preventing bypass graft failure. To do this we are analysing the behaviour of sections of arteries and veins and also extracting and growing different cell types from within blood vessels using a process termed tissue culture. The programme of research involves several different studies examining different aspects of bypass graft failure and vascular disease processes.

Why have I been chosen?

After your bypass surgery there may be small pieces of arteries or veins left over which were not required for your treatment and which would otherwise be discarded. We are inviting you to allow us to use these excess pieces for our research.

What are the possible disadvantages and risks of taking part?

There are no disadvantages to giving your permission for us to use these surplus pieces of tissue for research. It will not affect your surgery or your subsequent treatment in any way.

What are the possible benefits of taking part?
There are no personal benefits to you in taking part and your decision will not affect your treatment in anyway. However, we hope that the information we get from this research may help to improve the outcome of bypass surgery for future patients.

What will happen to my samples?
We will analyse samples of the graft to determine either how the individual cells or the whole graft responds to stimuli. ALL YOUR SAMPLES WILL BE CODED and only this unique code will be used to identify the sample in the laboratory. Identifiable information such as hospital number and date of birth will be stored in a secure database. The tissue samples will be considered to be a gift to the Cardiothoracic Centre, which will act as a custodian of all of the samples obtained as a result of these studies. As part of the ongoing studies samples will be shared with researchers at the University of Liverpool and rarely a small amount of your sample will be provided to other researchers in the UK or other parts of the world. However, it is important to remember that this will only be identified by a unique code.

In the short-term, it is unlikely that the samples will be of any commercial value to the Cardiothoracic Centre or University of Liverpool – any commercial value in the future is likely to be due to findings in groups of patients rather than from samples from a single patient.

Do I have to take part?
It is up to you to decide whether or not to give permission. Whether or not you give permission for these excess pieces of tissue to be used in our research will not affect the standard of care you receive or your treatment in any way.

What do I have to do?

If you decide to give your permission you will be given this information sheet to keep and be asked to sign a consent form. If you decide not to give your permission this is entirely at your discretion and you do not have to give a reason.

Will my taking part in this study be kept confidential?
If you consent to give permission for excess tissue to be used in research, all information that is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the study?
The results of our research will be published in scientific journals over the next few years. You will not be identified in any report or publication.

Who is organising and funding the Research?
The programme of research is being organised by Doctors and Scientists in the Research Department of the Cardiothoracic Centre. Some of the studies within the programme are funded by NHS Research funding and others by charitable Institutions

Who has reviewed the study?
The Liverpool Research Ethics Committee has approved the following studies within the research programme:

1. Nitric Oxide Generation And Mechanisms For Scavenging Reactive Oxygen Species In Arterial And Venous Graft Endothelium.
2. Prevention of spasm in radial artery grafts

Contacts for further information
If you should require any further information you can contact members of the research team:

Mr Michael Shackcloth, Specialist Registrar, Cardiac Surgery (please contact CTC switchboard)

Dr Paul Browning, Principal Clinical Scientist: Tel 0151 293 222

CONSENT FORM

RESEARCH INTO VASCULAR DISEASE AND BYPASS GRAFT FAILURE AT THE CARDIOTHORACIC CENTRE

Please initial box

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from the Cardiothoracic Centre or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

Bibliography

(1) Keogh BE, On behalf of The Society of Cardiothoracic Surgeons of Great Britain and Ireland. National Adult Cardiac Surgical Database Report 2000 - 2001. 72. 1-10-2002.

Ref Type: Generic

(2) Bridgewater B, Grayson AD, Brooks N, Grotte G, Fabri BM, Au J, et al. Has the publication of cardiac surgery outcome data been associated with changes in practice in northwest England: an analysis of 25,730 patients undergoing CABG surgery under 30 surgeons over eight years.[see comment]. Heart 2007 Jun;93(6):744-8.

(3) Vineberg A, Miller G. Internal Mammary Coronary Anastomosis in the Surgical Treatment of Coronary Artery Insufficiency. Canadian Medical Association Journal 64, 204-210. 1951.

Ref Type: Journal (Full)

(4) Murray G, Porcheron R, Hilario J. Anastomosis of a Systemic Artery to the Coronary Artery. Canadian Medical Association Journal 71, 594-598. 1954.

Ref Type: Journal (Full)

(5) Longmire WP, Cannon JA, Kattus AA. Direct -Vision Coronary Endarterectomy For Angina Pectoris. New England Journal Of Medicine 259, 993-999. 20-11-1968.

Ref Type: Generic

(6) Westerby S, Bosher C. Surgery for coronary artery disease. Landmarks in Cardiac Surgery. 2000, p. 187-222.

(7) Favaloro RG. Saphenous vein autograft replacement of severe segmental coronary artery occlusion: operative technique. Annals of Thoracic Surgery 1968 Apr;5(4):334-9.

(8) Green GE, Stertzer SH, Reppert EH. Coronary arterial bypass grafts. Annals of Thoracic Surgery 1968 May;5(5):443-50.

(9) Loop FD, Lytle BW, Cosgrove DM, Stewart RW, Goormastic M, Williams GW, et al. Influence of the internal-mammary-artery graft on 10-year survival and other cardiac events. New England Journal Of Medicine 1986 Jan 2;314(1):1-6.

(10) Cameron A, Kemp HG, Jr., Green GE. Bypass surgery with the internal mammary artery graft: 15 year follow-up. Circulation 1986 Nov;74(5 Pt 2):III30-III36.

(11) Zeff RH, Kongtahworn C, Iannone LA, Gordon DF, Brown TM, Phillips SJ, et al. Internal mammary artery versus saphenous vein graft to the left anterior descending coronary artery: prospective randomized study with 10-year follow-up. Annals of Thoracic Surgery 1988 May;45(5):533-6.

(12) Yusuf S, Zucker D, Peduzzi P, Fisher LD, Takaro T, Kennedy JW, et al. Effect of coronary artery bypass graft surgery on survival: overview of 10-year results from randomised trials by the Coronary Artery Bypass Graft Surgery Trialists Collaboration.[see comment][erratum appears in Lancet 1994 Nov 19;344(8934):1446]. Lancet 1994 Aug 27;344(8922):563-70.

(13) Taggart DP, D'Amico R, Altman DG. Effect of arterial revascularisation on survival: a systematic review of studies comparing bilateral and single internal mammary arteries.[see comment]. Lancet 2001 Sep 15;358(9285):870-5.

(14) Calafiore AM, Contini M, Vitolla G, Di Mauro M, Mazzei V, Teodori G, et al. Bilateral internal thoracic artery grafting: long-term clinical and angiographic results of in situ versus Y grafts. Journal of Thoracic & Cardiovascular Surgery 2000 Nov;120(5):990-6.

(15) Endo M, Nishida H, Tomizawa Y, Kasanuki H. Benefit of bilateral over single internal mammary artery grafts for multiple coronary artery bypass grafting.[see comment]. Circulation 2001 Oct 30;104(18):2164-70.

(16) Fitzgibbon GM, Kafka HP, Leach AJ, Keon WJ, Hooper GD, Burton JR. Coronary bypass graft fate and patient outcome: angiographic follow-up of 5,065 grafts related

to survival and reoperation in 1,388 patients during 25 years.[see comment]. Journal of the American College of Cardiology 1996 Sep;28(3):616-26.

(17) Lytle BW, Blackstone EH, Loop FD, Houghtaling PL, Arnold JH, Akhrass R, et al. Two internal thoracic artery grafts are better than one. Journal of Thoracic & Cardiovascular Surgery 1999 May;117(5):855-72.

(18) Berreklouw E, Rademakers PP, Koster JM, van Leur L, van der Wielen BJ, Westers P. Better ischemic event-free survival after two internal thoracic artery grafts: 13 years of follow-up. Annals of Thoracic Surgery 2001 Nov;72(5):1535-41.

(19) Buxton BF, Komeda M, Fuller JA, Gordon I. Bilateral internal thoracic artery grafting may improve outcome of coronary artery surgery. Risk-adjusted survival. Circulation 1998 Nov 10;98(19 Suppl):II1-II6.

(20) Pick AW, Orszulak TA, Anderson BJ, Schaff HV. Single versus bilateral internal mammary artery grafts: 10-year outcome analysis.[see comment]. Annals of Thoracic Surgery 1997 Sep;64(3):599-605.

(21) Endo M, Nishida H, Tomizawa Y, Kasanuki H. Benefit of bilateral over single internal mammary artery grafts for multiple coronary artery bypass grafting.[comment]. Circulation 2001 Oct 30;104(18):2164-70.

(22) Lu JC, Grayson AD, Jha P, Srinivasan AK, Fabri BM. Risk factors for sternal wound infection and mid-term survival following coronary artery bypass surgery. European Journal of Cardio-Thoracic Surgery 2003 Jun;23(6):943-9.

(23) Matsa M, Paz Y, Gurevitch J, Shapira I, Kramer A, Pevny D, et al. Bilateral skeletonized internal thoracic artery grafts in patients with diabetes mellitus.[see comment]. Journal of Thoracic & Cardiovascular Surgery 2001 Apr;121(4):668-74.

(24) Taggart DP. Respiratory dysfunction after cardiac surgery: effects of avoiding cardiopulmonary bypass and the use of bilateral internal mammary arteries. European Journal of Cardio-Thoracic Surgery 2000 Jul;18(1):31-7.

(25) Nwasokwa ON. Coronary artery bypass graft disease. [Review] [148 refs]. Annals of Internal Medicine 1995 Oct 1;123(7):528-45.

(26) Society of Cardiothoracic Surgeons of Great Britain and Ireland. National Adult Cardiac Surgical Database Report 1999-2000. 2001.

Ref Type: Pamphlet

(27) Bailey CP, Hirose T, Aventura A, Yamamoto N, Brancato R, Vera C, et al. Revascularization of the ischemic posterior myocardium. Diseases of the Chest 1967 Sep;52(3):273-85.

(28) Mills NL, Everson CT. Right gastroepiploic artery: a third arterial conduit for coronary artery bypass.[see comment]. Annals of Thoracic Surgery 1989 May;47(5):706-11.

(29) Pym J, Brown PM, Charrette EJ, Parker JO, West RO. Gastroepiploic-coronary anastomosis. A viable alternative bypass graft. Journal of Thoracic & Cardiovascular Surgery 1987 Aug;94(2):256-9.

(30) Suma H, Fukumoto H, Takeuchi A. Coronary artery bypass grafting by utilizing in situ right gastroepiploic artery: basic study and clinical application. Annals of Thoracic Surgery 1987 Oct;44(4):394-7.

(31) Manapat AE, McCarthy PM, Lytle BW, Taylor PC, Loop FD, Stewart RW, et al. Gastroepiploic and inferior epigastric arteries for coronary artery bypass. Early results and evolving applications. Circulation 1994 Nov;90(5 Pt 2):II144-II147.

(32) Puig LB, Ciongolli W, Cividanes GV, Dontos A, Kopel L, Bittencourt D, et al. Inferior epigastric artery as a free graft for myocardial revascularization.[see comment]. Journal of Thoracic & Cardiovascular Surgery 1990 Feb;99(2):251-5.

(33) Puig LB, Ciongoli W, Cividanes GV, Teofilo JS, Dontof AC, Fiorelli AI, et al. [Lower epigastric artery as a free graft. A new alternative in direct myocardial revascularization]. [Portuguese]. Arquivos Brasileiros de Cardiologia 1988 Apr;50(4):259-61.

(34) Calafiore AM, Di Giammarco G, Teodori G, D'Annunzio E, Vitolla G, Fino C, et al. Radial artery and inferior epigastric artery in composite grafts: improved midterm angiographic results. Annals of Thoracic Surgery 1995 Sep;60(3):517-23.

(35) Barner HB, Naunheim KS, Fiore AC, Fischer VW, Harris HH. Use of the inferior epigastric artery as a free graft for myocardial revascularization.[see comment]. Annals of Thoracic Surgery 1991 Sep;52(3):429-36.

(36) Carpentier A, Guermonprez JL, Deloche A, Frechette C, DuBost C. The aorta-to-coronary radial artery bypass graft. A technique avoiding pathological changes in grafts. Annals of Thoracic Surgery 1973 Aug;16(2):111-21.

(37) Geha AS, Krone RJ, McCormick JR, Baue AE. Selection of coronary bypass. Anatomic, physiological, and angiographic considerations of vein and mammary artery grafts. J Thorac Cardiovasc Surg 1975 Sep 1;70(3):414-31.

(38) Curtis JJ, Stoney WS, Alford WC, Jr., Burrus GR, Thomas CS, Jr. Intimal hyperplasia. A cause of radial artery aortocoronary bypass graft failure. Annals of Thoracic Surgery 1975 Dec;20(6):628-35.

(39) Fisk RL, Brooks CH, Callaghan JC, Dvorkin J. Experience with the radial artery graft for coronary artery bypass. Annals of Thoracic Surgery 1976 Jun;21(6):513-8.

(40) Acar C, Jebara VA, Portoghese M, Beyssen B, Pagny JY, Grare P, et al. Revival of the radial artery for coronary artery bypass grafting.[see comment]. Annals of Thoracic Surgery 1992 Oct;54(4):652-9.

(41) He GW, Yang CQ. Use of verapamil and nitroglycerin solution in preparation of radial artery for coronary grafting. Annals of Thoracic Surgery 1996 Feb;61(2):610-4.

(42) Brodman RF, Frame R, Camacho M, Hu E, Chen A, Hollinger I. Routine use of unilateral and bilateral radial arteries for coronary artery bypass graft surgery.[comment]. Journal of the American College of Cardiology 1996 Oct;28(4):959-63.

(43) Possati G, Gaudino M, Alessandrini F, Luciani N, Glieca F, Trani C, et al. Midterm clinical and angiographic results of radial artery grafts used for myocardial revascularization.[comment]. Journal of Thoracic & Cardiovascular Surgery 1998 Dec;116(6):1015-21.

(44) Possati G, Gaudino M, Prati F, Alessandrini F, Trani C, Glieca F, et al. Long-term results of the radial artery used for myocardial revascularization. Circulation 2003 Sep 16;108(11):1350-4.

(45) Acar C, Ramsheyi A, Pagny JY, Jebara V, Barrier P, Fabiani JN, et al. The radial artery for coronary artery bypass grafting: clinical and angiographic results at five years.[comment]. Journal of Thoracic & Cardiovascular Surgery 1998 Dec;116(6):981-9.

(46) Buxton BF, Raman JS, Ruengsakulrach P, Gordon I, Rosalion A, Bellomo R, et al. Radial artery patency and clinical outcomes: five-year interim results of a randomized trial. Journal of Thoracic & Cardiovascular Surgery 2003 Jun;125(6):1363-71.

(47) Tatoulis J, Royse AG, Buxton BF, Fuller JA, Skillington PD, Goldblatt JC, et al. The radial artery in coronary surgery: a 5-year experience--clinical and angiographic results. Annals of Thoracic Surgery 2002 Jan;73(1):143-7.

(48) Iaco AL, Teodori G, Di Giammarco G, Di Mauro M, Storto L, Mazzei V, et al. Radial artery for myocardial revascularization: long-term clinical and angiographic results. Annals of Thoracic Surgery 2001 Aug;72(2):464-8.

(49) da Costa FD, da Costa IA, Poffo R, Abuchaim D, Gaspar R, Garcia L, et al. Myocardial revascularization with the radial artery: a clinical and angiographic study. Annals of Thoracic Surgery 1996 Aug;62(2):475-9.

(50) Taggart DP. The radial artery as a conduit for coronary artery bypass grafting. Heart (British Cardiac Society) 1999 Oct;82(4):409-10.

(51) Anyanwu AC, Saeed I, Bustami M, Ilsley C, Yacoub MH, Amrani M. Does routine use of the radial artery increase complexity or morbidity of coronary bypass surgery? Annals of Thoracic Surgery 2001 Feb;71(2):555-9.

(52) Saeed I, Anyanwu AC, Yacoub MH, Amrani M. Subjective patient outcomes following coronary artery bypass using the radial artery: results of a cross-sectional survey of harvest site complications and quality of life. European Journal of Cardio-Thoracic Surgery 2001 Dec;20(6):1142-6.

(53) Royse AG, Royse CF, Shah P, Williams A, Kaushik S, Tatoulis J. Radial artery harvest technique, use and functional outcome. European Journal of Cardio-Thoracic Surgery 1999 Feb;15(2):186-93.

(54) Nunoo-Mensah J. An unexpected complication after harvesting of the radial artery for coronary artery bypass grafting.[comment]. Annals of Thoracic Surgery 1998 Sep;66(3):929-31.

(55) He GW, Yang CQ. Comparison among arterial grafts and coronary artery. An attempt at functional classification.[see comment]. Journal of Thoracic & Cardiovascular Surgery 1995 Apr;109(4):707-15.

(56) Cable DG, Caccitolo JA, Pearson PJ, O'Brien T, Mullany CJ, Daly RC, et al. New approaches to prevention and treatment of radial artery graft vasospasm. Circulation 1998 Nov 10;98(19 Suppl):II15-II21.

(57) Manasse E, Sperti G, Suma H, Canosa C, Kol A, Martinelli L, et al. Use of the radial artery for myocardial revascularization.[comment]. Annals of Thoracic Surgery 1996 Oct;62(4):1076-82.

(58) Chen AH, Nakao T, Brodman RF, Greenberg M, Charney R, Menegus M, et al. Early postoperative angiographic assessment of radial grafts used for coronary artery bypass grafting. Journal of Thoracic & Cardiovascular Surgery 1996 Jun;111(6):1208-12.

(59) Rodriguez E, Ormont ML, Lambert EH, Needleman L, Halpern EJ, Diehl JT, et al. The role of preoperative radial artery ultrasound and digital plethysmography prior to

coronary artery bypass grafting. European Journal of Cardio-Thoracic Surgery 2001 Feb;19(2):135-9.

(60) Wright CB, Barner HB, Gao A, Obial R, Bandy B, Perry L, et al. The advantages of the Harmonic Scalpel for the harvesting of radial arteries for coronary artery bypass. Heart Surgery Forum 2001;4(3):226-9.

(61) Birdi I, Ritchie AJ. Intraoperative confirmation of ulnar collateral blood flow during radial artery harvesting using the "squirt test". Annals of Thoracic Surgery 2002 Jul;74(1):271-2.

(62) Ruengsakulrach P, Eizenberg N, Fahrer C, Fahrer M, Buxton BF. Surgical implications of variations in hand collateral circulation: anatomy revisited. Journal of Thoracic & Cardiovascular Surgery 2001 Oct;122(4):682-6.

(63) van Son JA, Smedts F, Vincent JG, van Lier HJ, Kubat K. Comparative anatomic studies of various arterial conduits for myocardial revascularization. Journal of Thoracic & Cardiovascular Surgery 1990 Apr;99(4):703-7.

(64) Chester AH, Marchbank AJ, Borland JA, Yacoub MH, Taggart DP. Comparison of the morphologic and vascular reactivity of the proximal and distal radial artery. Annals of Thoracic Surgery 1998 Dec;66(6):1972-6.

(65) Dietl CA, Benoit CH. Radial artery graft for coronary revascularization: technical considerations. Annals of Thoracic Surgery 1995 Jul;60(1):102-9.

(66) Geha AS, Hammond GL, Stephan RN, Kleiger RK, Krone RJ. Long-term outcome of revascularization of the anterior coronary arteries with crossed double internal mammary versus saphenous vein grafts. Surgery 1987 Oct;102(4):667-73.

(67) Sims FH. A comparison of coronary and internal mammary arteries and implications of the results in the etiology of arteriosclerosis. American Heart Journal 1983 Apr;105(4):560-6.

(68) He GW, Yang CQ. Radial artery has higher receptor-mediated contractility but similar endothelial function compared with mammary artery. Annals of Thoracic Surgery 1997 May;63(5):1346-52.

(69) Chaikhouni A, Crawford FA, Kochel PJ, Olanoff LS, Halushka PV. Human internal mammary artery produces more prostacyclin than saphenous vein. Journal of Thoracic & Cardiovascular Surgery 1986 Jul;92(1):88-91.

(70) Hagiwara H, Ito T, Kamiya H, Akita T, Usui A, Ueda Y. Mid-term structural change in the radial artery grafts after coronary artery bypass grafting. Annals of Thoracic Surgery 2004 Mar;77(3):805-10.

(71) Walts AE, Fishbein MC, Matloff JM. Thrombosed, ruptured atheromatous plaques in saphenous vein coronary artery bypass grafts: ten years' experience. American Heart Journal 1987 Oct;114(4 Pt 1):718-23.

(72) He GW, Yang CQ, Starr A. Overview of the nature of vasoconstriction in arterial grafts for coronary operations. Annals of Thoracic Surgery 1995 Mar;59(3):676-83.

(73) Borland JA, Chester AH, Rooker SJ, Wharton J, Davie N, Amrani M, et al. Expression and function of angiotensin converting enzyme, chymase, and angiotensin II in the human radial artery and internal thoracic artery. Annals of Thoracic Surgery 2000 Dec;70(6):2054-63.

(74) Chanda J, Brichkov I, Canver CC. Prevention of radial artery graft vasospasm after coronary bypass. Annals of Thoracic Surgery 2000 Dec;70(6):2070-4.

(75) Chardigny C, Jebara VA, Acar C, Descombes JJ, Verbeuren TJ, Carpentier A, et al. Vasoreactivity of the radial artery. Comparison with the internal mammary and gastroepiploic arteries with implications for coronary artery surgery. Circulation 1993 Nov;88(5 Pt 2):II115-II127.

(76) He GW, Yang CQ. Characteristics of adrenoceptors in the human radial artery: clinical implications. Journal of Thoracic & Cardiovascular Surgery 1998 May;115(5):1136-41.

(77) He GW, Buxton B, Rosenfeldt FL, Wilson AC, Angus JA. Weak beta-adrenoceptor-mediated relaxation in the human internal mammary artery. Journal of Thoracic & Cardiovascular Surgery 1989 Feb;97(2):259-66.

(78) Robertson RM, Wood AJ, Vaughn WK, Robertson D. Exacerbation of vasotonic angina pectoris by propranolol. Circulation 1982 Feb;65(2):281-5.

(79) Langer SZ, Shepperson NB, Massingham R. Preferential noradrenergic innervation of alpha-adrenergic receptors in vascular smooth muscle. Hypertension 1981 May;3(3 Pt 2):I112-I118.

(80) Langer SZ, Hicks PE. Alpha-adrenoreceptor subtypes in blood vessels: physiology and pharmacology. [Review] [75 refs]. Journal of Cardiovascular Pharmacology 1984;6 Suppl 4:S547-S558.

(81) Medgett IC, Hicks PE, Langer SZ. Smooth muscle alpha-2 adrenoceptors mediate vasoconstrictor responses to exogenous norepinephrine and to sympathetic stimulation to a greater extent in spontaneously hypertensive than in Wistar Kyoto rat tail arteries. Journal of Pharmacology & Experimental Therapeutics 1984 Oct;231(1):159-65.

(82) Parkinson NA, Thom SM, Hughes AD, Sever PS, Mulvany MJ, Nielsen H. Neurally evoked responses of human isolated resistance arteries are mediated by both alpha 1- and alpha 2-adrenoceptors. British Journal of Pharmacology 1992 Jul;106(3):568-73.

(83) Williams M, Glennon RA, Timmermans PBMWM. α-adrenoreceptor. Receptor pharmacology and function., 1st ed. New York: Marcel Dekker; 1989, p. 173-205.

(84) Conant AR, Oo AY, Dashwood MR, Collard K, Chester MR, Dihmis WC, et al. Endothelin receptors in cultured and native human radial artery smooth muscle. Journal of Cardiovascular Pharmacology 2002 Jan;39(1):130-41.

(85) Desai ND, Fremes SE, Desai ND, Fremes SE. Radial artery conduit for coronary revascularization: as good as an internal thoracic artery?. [Review] [71 refs]. Current Opinion in Cardiology 2007 Nov;22(6):534-40.

(86) Rosenfeldt FL, He GW, Buxton BF, Angus JA, Rosenfeldt FL, He GW, et al. Pharmacology of coronary artery bypass grafts.[see comment]. [Review] [46 refs]. Ann Thorac Surg 1999 Mar;67(3):878-88.

(87) Apostolidou IA, Skubas NJ, Despotis GJ, Kallinteri E, Hogue CW, Lappas DG, et al. Occurrence of myocardial ischemia immediately after coronary revascularization using radial arterial conduits. Journal of Cardiothoracic & Vascular Anesthesia 2001 Aug;15(4):433-8.

(88) Kulik A, Rubens FD, Gunning D, Bourke ME, Mesana TG, Ruel M, et al. Radial artery graft treatment with phenoxybenzamine is clinically safe and may reduce perioperative myocardial injury.[see comment]. Ann Thorac Surg 2007 Feb;83(2):502-9.

(89) Taggart DP, Mathur MN, Ahmad I. Skeletonization of the radial artery: advantages over the pedicled technique.[comment]. Annals of Thoracic Surgery 2001 Jul;72(1):298-9.

(90) Oo AY, Conant AR, Chester MR, Dihmis WC, Simpson AW. Temperature changes stimulate contraction in the human radial artery and affect response to vasoconstrictors.[see comment]. Ann Thorac Surg 2007 Jan;83(1):126-32.

(91) Downing SW, Edmunds LH, Jr. Release of vasoactive substances during cardiopulmonary bypass.[see comment]. [Review] [76 refs]. Annals of Thoracic Surgery 1992 Dec;54(6):1236-43.

(92) Conant AR, Shackcloth MJ, Oo AY, Chester MR, Simpson AW, Dihmis WC, et al. Phenoxybenzamine treatment is insufficient to prevent spasm in the radial artery: the effect of other vasodilators. J Thorac Cardiovasc Surg 2003 Aug;126(2):448-54.

(93) Kuan P, Messenger JC, Ellestad MH, Kuan P, Messenger JC, Ellestad MH. Transient central diabetes insipidus after aortocoronary bypass operations. Am J Cardiol 1983 Dec 1;52(10):1181-3.

(94) Wei W, Yang CQ, Furnary A, He GW, Wei W, Yang CQ, et al. Greater vasopressin-induced vasoconstriction and inferior effects of nitrovasodilators and milrinone in the radial artery than in the internal thoracic artery. J Thorac Cardiovasc Surg 2005 Jan;129(1):33-40.

(95) Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells.[see comment]. Nature 1988 Mar 31;332(6163):411-5.

(96) Vane JR, Anggard EE, Botting RM. Regulatory functions of the vascular endothelium. [Review] [118 refs]. New England Journal Of Medicine 1990 Jul 5;323(1):27-36.

(97) Bourque CW, Oliet SH, Richard D, Bourque CW, Oliet SH, Richard D. Osmoreceptors, osmoreception, and osmoregulation. [Review] [420 refs]. Frontiers in Neuroendocrinology 1994 Sep;15(3):231-74.

(98) Aisenbrey GA, Handelman WA, Arnold P, Manning M, Schrier RW, Aisenbrey GA, et al. Vascular effects of arginine vasopressin during fluid deprivation in the rat. Journal of Clinical Investigation 1981 Apr;67(4):961-8.

(99) Schwartz J, Reid IA, Schwartz J, Reid IA. Effect of vasopressin blockade on blood pressure regulation during hemorrhage in conscious dogs. Endocrinology 1981 Nov;109(5):1778-80.

(100) Khayyal MA, Eng C, Franzen D, Breall JA, Kirk ES, Khayyal MA, et al. Effects of vasopressin on the coronary circulation: reserve and regulation during ischemia. American Journal of Physiology 1985 Apr;248(4 Pt 2):H516-H522.

(101) Wei W, Floten HS, He GW, Wei W, Floten HS, He GW. Interaction between vasodilators and vasopressin in internal mammary artery and clinical significance. Ann Thorac Surg 2002 Feb;73(2):516-22.

(102) Maturi MF, Martin SE, Markle D, Maxwell M, Burruss CR, Speir E, et al. Coronary vasoconstriction induced by vasopressin. Production of myocardial ischemia in dogs by constriction of nondiseased small vessels. Circulation 1991 Jun;83(6):2111-21.

(103) Medina P, Acuna A, Martinez-Leon JB, Otero E, Vila JM, Aldasoro M, et al. Arginine vasopressin enhances sympathetic constriction through the V1 vasopressin receptor in human saphenous vein. Circulation 1998 Mar 10;97(9):865-70.

(104) Argenziano M, Chen JM, Choudhri AF, Cullinane S, Garfein E, Weinberg AD, et al. Management of vasodilatory shock after cardiac surgery: identification of predisposing factors and use of a novel pressor agent.[see comment]. J Thorac Cardiovasc Surg 1998 Dec;116(6):973-80.

(105) Chernow B, Roth BL, Chernow B, Roth BL. Pharmacologic manipulation of the peripheral vasculature in shock: clinical and experimental approaches. [Review] [116 refs]. Circulatory Shock 1986;18(2):141-55.

(106) Reyes AT, Frame R, Brodman RF. Technique for harvesting the radial artery as a coronary artery bypass graft.[comment]. Annals of Thoracic Surgery 1995 Jan;59(1):118-26.

(107) He GW. Verapamil plus nitroglycerin solution maximally preserves endothelial function of the radial artery: comparison with papaverine solution. Journal of Thoracic & Cardiovascular Surgery 1998 Jun;115(6):1321-7.

(108) Lincoln TM, Cornwell TL. Intracellular cyclic GMP receptor proteins. [Review] [106 refs]. FASEB Journal 1993 Feb 1;7(2):328-38.

(109) Polson JB, Strada SJ. Cyclic nucleotide phosphodiesterases and vascular smooth muscle. [Review] [87 refs]. Annual Review of Pharmacology & Toxicology 1996;36:403-27.

(110) Armstrong PW, Armstrong JA, Marks GS. Pharmacokinetic-hemodynamic studies of intravenous nitroglycerin in congestive cardiac failure. Circulation 1980 Jul;62(1):160-6.

(111) Shapira OM, Xu A, Vita JA, Aldea GS, Shah N, Shemin RJ, et al. Nitroglycerin is superior to diltiazem as a coronary bypass conduit vasodilator. Journal of Thoracic & Cardiovascular Surgery 1999 May;117(5):906-11.

(112) Shapira OM, Alkon JD, Macron DS, Keaney JF, Jr., Vita JA, Aldea GS, et al. Nitroglycerin is preferable to diltiazem for prevention of coronary bypass conduit spasm.[comment]. Annals of Thoracic Surgery 2000 Sep;70(3):883-8.

(113) Elkayam U, Kulick D, McIntosh N, Roth A, Hsueh W, Rahimtoola SH. Incidence of early tolerance to hemodynamic effects of continuous infusion of nitroglycerin in patients with coronary artery disease and heart failure. Circulation 1987 Sep;76(3):577-84.

(114) Munzel T, Heitzer T, Kurz S, Harrison DG, Luhman C, Pape L, et al. Dissociation of coronary vascular tolerance and neurohormonal adjustments during long-term nitroglycerin therapy in patients with stable coronary artery disease. Journal of the American College of Cardiology 1996 Feb;27(2):297-303.

(115) Caramori PR, Adelman AG, Azevedo ER, Newton GE, Parker AB, Parker JD. Therapy with nitroglycerin increases coronary vasoconstriction in response to acetylcholine. Journal of the American College of Cardiology 1998 Dec;32(7):1969-74.

(116) Munzel T, Li H, Mollnau H, Hink U, Matheis E, Hartmann M, et al. Effects of long-term nitroglycerin treatment on endothelial nitric oxide synthase (NOS III) gene expression, NOS III-mediated superoxide production, and vascular NO bioavailability. Circulation Research 2000 Jan 7;86(1):E7-E12.

(117) Mussa S, Guzik TJ, Black E, Dipp MA, Channon KM, Taggart DP, et al. Comparative efficacies and durations of action of phenoxybenzamine, verapamil/nitroglycerin solution, and papaverine as topical antispasmodics for radial artery coronary bypass grafting. J Thorac Cardiovasc Surg 2003 Dec;126(6):1798-805.

(118) Abernethy DR, Schwartz JB. Calcium-antagonist drugs. [Review] [102 refs]. New England Journal Of Medicine 1999 Nov 4;341(19):1447-57.

(119) Barner HB. The continuing evolution of arterial conduits. Ann Thorac Surg 1999 Sep;68(3:Suppl):Suppl-8.

(120) Shapira OM, Alkon JD, Aldea GS, Madera F, Lazar HL, Shemin RJ. Clinical outcomes in patients undergoing coronary artery bypass grafting with preferred use of the radial artery. Journal of Cardiac Surgery 1997 Nov;12(6):381-8.

(121) He GW, Yang CQ. Comparative study on calcium channel antagonists in the human radial artery: clinical implications. Journal of Thoracic & Cardiovascular Surgery 2000 Jan;119(1):94-100.

(122) Ferrari R. Major differences among the three classes of calcium antagonists. [Review] [177 refs]. European Heart Journal 1997 Jan;18 Suppl A:A56-A70.

(123) Bond BR, Zellner JL, Dorman BH, Multani MM, Kratz JM, Crumbley AJ, III, et al. Differential effects of calcium channel antagonists in the amelioration of radial artery vasospasm.[comment]. Annals of Thoracic Surgery 2000 Apr;69(4):1035-40.

(124) He GW, Rosenfeldt FL, Buxton BF, Angus JA. Reactivity of human isolated internal mammary artery to constrictor and dilator agents. Implications for treatment of internal mammary artery spasm. Circulation 1989 Sep;80(3 Pt 1):I141-I150.

(125) Henry PD. Comparative pharmacology of calcium antagonists: nifedipine, verapamil and diltiazem. [Review] [113 refs]. Am J Cardiol 1980 Dec 1;46(6):1047-58.

(126) Saadjian A, Philip-Joet F, Paganelli F, Saadjian M, Levy S. [Acute effects of nicardipine on the vascular reactivity of oxygen in patients with respiratory insufficiency and pulmonary hypertension]. [Review] [30 refs] [French]. Arch Mal Coeur Vaiss 1993 Jun;86(6):899-906.

(127) He GW, Rosenfeldt FL, Angus JA. Pharmacological relaxation of the saphenous vein during harvesting for coronary artery bypass grafting. Annals of Thoracic Surgery 1993 May;55(5):1210-7.

(128) He GW, Buxton BF, Rosenfeldt FL, Angus JA, Tatoulis J. Pharmacologic dilatation of the internal mammary artery during coronary bypass grafting. Journal of Thoracic & Cardiovascular Surgery 1994 Jun;107(6):1440-4.

(129) Dipp MA, Nye PC, Taggart DP. Phenoxybenzamine is more effective and less harmful than papaverine in the prevention of radial artery vasospasm. European Journal of Cardio-Thoracic Surgery 2001 Apr;19(4):482-6.

(130) Huddart H, Saad KH. Papaverine-induced inhibition of electrical and mechanical activity and calcium movements of rat ileal smooth muscle. Journal of Experimental Biology 1980 Jun;86:99-114.

(131) Brading AF, Burdyga TV, Scripnyuk ZD. The effects of papaverine on the electrical and mechanical activity of the guinea-pig ureter. Journal of Physiology 1983 Jan;334:79-89.

(132) Cooper GJ, Gillot T, Parry EA, Kennedy A, Wilkinson GA. Papaverine injures the endothelium of the internal mammary artery. Cardiovascular Surgery 1995 Oct;3(5):553-5.

(133) Cracowski JL, Stanke-Labesque F, Chavanon O, Blin D, Mallion JM, Bessard G, et al. Vasorelaxant actions of enoximone, dobutamine, and the combination on human arterial coronary bypass grafts. Journal of Cardiovascular Pharmacology 1999 Nov;34(5):741-8.

(134) Liu JJ, Doolan LA, Xie B, Chen JR, Buxton BF. Direct vasodilator effect of milrinone, an inotropic drug, on arterial coronary bypass grafts. FANZCA .[see comment]. Journal of Thoracic & Cardiovascular Surgery 1997 Jan;113(1):108-13.

(135) Boldt J, Kling D, Dieterich HA, Marck P, Hempelmann G. The new phosphodiesterase inhibitor enoximone in patients following cardiac surgery--

pharmacokinetics and influence on parameters of coagulation. Intensive Care Medicine 1990;16(1):54-9.

(136) Cracowski JL, Chavanon O, Durand M, Borrel E, Devillier P, Mallion JM, et al. Effect of low-dose positive inotropic drugs on human internal mammary artery flow. Annals of Thoracic Surgery 1997 Dec;64(6):1742-6.

(137) Salmenpera M, Levy JH. The in vitro effects of phosphodiesterase inhibitors on the human internal mammary artery. Anesthesia & Analgesia 1996 May;82(5):954-7.

(138) Bailey JM, Levy JH, Kikura M, Szlam F, Hug CC, Jr. Pharmacokinetics of intravenous milrinone in patients undergoing cardiac surgery. Anesthesiology 1994 Sep;81(3):616-22.

(139) He GW, Yang CQ. Vasorelaxant effect of phosphodiesterase-inhibitor milrinone in the human radial artery used as coronary bypass graft. Journal of Thoracic & Cardiovascular Surgery 2000 May;119(5):1039-45.

(140) Boolell M, Allen MJ, Ballard SA, Gepi-Attee S, Muirhead GJ, Naylor AM, et al. Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. International Journal of Impotence Research 1996 Jun;8(2):47-52.

(141) Lincoln TM, Cornwell TL. Intracellular cyclic GMP receptor proteins. [Review] [106 refs]. FASEB Journal 1993 Feb 1;7(2):328-38.

(142) Medina P, Segarra G, Martinez-Leon JB, Vila JM, Aldasoro M, Otero E, et al. Relaxation induced by cGMP phosphodiesterase inhibitors sildenafil and zaprinast in human vessels. Annals of Thoracic Surgery 2000 Oct;70(4):1327-31.

(143) Taggart DP, Dipp M, Mussa S, Nye PC. Phenoxybenzamine prevents spasm in radial artery conduits for coronary artery bypass grafting. Journal of Thoracic & Cardiovascular Surgery 2000 Oct;120(4):815-7.

(144) Velez DA, Morris CD, Muraki S, Budde JM, Otto RN, Zhao ZQ, et al. Brief pretreatment of radial artery conduits with phenoxybenzamine prevents vasoconstriction long term. Annals of Thoracic Surgery 2001 Dec;72(6):1977-84.

(145) Corvera JS, Morris CD, Budde JM, Velez DA, Puskas JD, Lattouf OM, et al. Pretreatment with phenoxybenzamine attenuates the radial artery's vasoconstrictor response to {alpha}-adrenergic stimuli. J Thorac Cardiovasc Surg 2003 Nov 1;126(5):1549-54.

(146) Mussa S, Guzik TJ, Black E, Dipp MA, Channon KM, Taggart DP. Comparative efficacies and durations of action of phenoxybenzamine, verapamil/nitroglycerin solution, and papaverine as topical antispasmodics for radial artery coronary bypass grafting. J Thorac Cardiovasc Surg 2003 Dec;126(6):1798-805.

(147) Mussa S, Prior T, Alp N, Wood K, Channon KM, Taggart DP, et al. Duration of action of antispasmodic agents: novel use of a mouse model as an in vivo pharmacological assay. Eur J Cardiothorac Surg 2004 Nov;26(5):988-94.

(148) Ning YM, Sanchez ER. Evidence for a functional interaction between calmodulin and the glucocorticoid receptor. Biochemical & Biophysical Research Communications 1995 Mar 8;208(1):48-54.

(149) Orallo F. Regulation of cytosolic calcium levels in vascular smooth muscle. [Review] [267 refs]. Pharmacology & Therapeutics 1996;69(3):153-71.

(150) Mussa S, Choudhary BP, Taggart DP. Radial artery conduits for coronary artery bypass grafting: Current perspective. J Thorac Cardiovasc Surg 2005 Feb 1;129(2):250-3.

(151) Taylor KM, Bain WH, Russell M, Brannan JJ, Morton IJ. Peripheral vascular resistance and angiotensin II levels during pulsatile and no-pulsatile cardiopulmonary bypass. Thorax 1979 Oct;34(5):594-8.

(152) Li HT, Long CS, Gray MO, Rokosh DG, Honbo NY, Karliner JS. Cross talk between angiotensin AT1 and alpha 1-adrenergic receptors: angiotensin II downregulates alpha

1a-adrenergic receptor subtype mRNA and density in neonatal rat cardiac myocytes. Circulation Research 1997 Sep;81(3):396-403.

(153) Fujimoto S. Effects of pimobendan, its active metabolite UD-CG 212, and milrinone on isolated blood vessels. European Journal of Pharmacology 1994 Nov 24;265(3):159-66.

(154) He GW, Yang CQ. Inhibition of vasoconstriction by potassium channel opener aprikalim in human conduit arteries used as bypass grafts. British Journal of Clinical Pharmacology 1997 Oct;44(4):353-9.

(155) Benetos A, Gautier S, Lafleche A, Topouchian J, Frangin G, Girerd X, et al. Blockade of angiotensin II type 1 receptors: effect on carotid and radial artery structure and function in hypertensive humans. Journal of Vascular Research 2000 Jan;37(1):8-15.

(156) He GW, Yang CQ. Inhibition of vasoconstriction by the thromboxane A2 antagonist GR32191B in the human radial artery. British Journal of Clinical Pharmacology 1999 Aug;48(2):207-15.

(157) Weber R, Pechere-Bertschi A, Hayoz D, Gerc V, Brouard R, Lahmy JP, et al. Effects of SR 49059, a new orally active and specific vasopressin V1 receptor antagonist, on vasopressin-induced vasoconstriction in humans.[erratum appears in Hypertension 1998 Feb;31(2):723]. Hypertension 1997 Nov;30(5):1121-7.

(158) Batchelor TJ, Sadaba JR, Ishola A, Pacaud P, Munsch CM, Beech DJ, et al. Rho-kinase inhibitors prevent agonist-induced vasospasm in human internal mammary artery. Br J Pharmacol 2001 Jan;132(1):302-8.

(159) Frei B, Stocker R, Ames BN. Antioxidant defenses and lipid peroxidation in human blood plasma. Proceedings of the National Academy of Sciences of the United States of America 1988 Dec;85(24):9748-52.

(160) Hamabe A, Takase B, Uehata A, Kurita A, Ohsuzu F, Tamai S. Impaired endothelium-dependent vasodilation in the brachial artery in variant angina pectoris

and the effect of intravenous administration of vitamin C. Am J Cardiol 2001 May 15;87(10):1154-9.

(161) Gokce N, Keaney JF, Jr., Frei B, Holbrook M, Olesiak M, Zachariah BJ, et al. Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease. Circulation 1999 Jun 29;99(25):3234-40.

(162) Drossos GE, Toumpoulis IK, Katritsis DG, Ioannidis JP, Kontogiorgi P, Svarna E, et al. Is vitamin C superior to diltiazem for radial artery vasodilation in patients awaiting coronary artery bypass grafting? Journal of Thoracic & Cardiovascular Surgery 2003 Feb;125(2):330-5.

(163) Mangoush O, Athanasiou T, Nakamura K, Johnson P, Smoienski R, Sarathchandra P, et al. Antioxidant properties of the internal thoracic artery and the radial artery. Heart, Lung & Circulation 2008 Feb;17(1):40-7.

(164) Cable DG, Caccitolo JA, Pearson PJ, O'Brien T, Mullany CJ, Daly RC, et al. New approaches to prevention and treatment of radial artery graft vasospasm. Circulation 1998 Nov 10;98(19 Suppl):II15-II21.

(165) Downing SW, Edmunds LH, Jr., Downing SW, Edmunds LHJ. Release of vasoactive substances during cardiopulmonary bypass.[see comment]. [Review] [76 refs]. Ann Thorac Surg 1992 Dec;54(6):1236-43.

(166) Soliman AS, Tackett RL. Altered responsiveness of saphenous vein grafts to norepinephrine and tyramine: relation to tissue catecholamine stores. J Cardiovasc Pharmacol 1991 Jan;17(1):154-7.

(167) Hamilton CA, Dalrymple HW, Reid JL, Sumner DJ. The recovery of alpha-adrenoceptor function and binding sites after phenoxybenzamine. An index of receptor turnover? Naunyn-Schmiedebergs Archives of Pharmacology 1984 Jan;325(1):34-41.

(168) Hu ZW, Shi XY, Okazaki M, Hoffman BB. Angiotensin II induces transcription and expression of alpha 1-adrenergic receptors in vascular smooth muscle cells. American Journal of Physiology 1995 Mar;268(3:Pt 2):t-14.

(169) Borland JA, Chester AH, Rooker SJ, Wharton J, Davie N, Amrani M, et al. Expression and function of angiotensin converting enzyme, chymase, and angiotensin II in the human radial artery and internal thoracic artery. Ann Thorac Surg 2000 Dec;70(6):2054-63.

(170) He GW, Yang CQ, He GW, Yang CQ. Radial artery has higher receptor-mediated contractility but similar endothelial function compared with mammary artery. Ann Thorac Surg 1997 May;63(5):1346-52.

(171) Dunser MW, Ohlbauer M, Rieder J, Zimmermann I, Ruatti H, Schwabegger AH, et al. Critical care management of major hydrofluoric acid burns: a case report, review of the literature, and recommendations for therapy. [Review] [84 refs]. Burns 2004 Jun;30(4):391-8.

(172) Hait WN, Glazer L, Kaiser C, Cross J, Kennedy KA, Hait WN, et al. Pharmacological properties of fluphenazine-mustard, an irreversible calmodulin antagonist. Molecular Pharmacology 1987 Sep;32(3):404-9.

(173) Sunagawa M, Kosugi T, Nakamura M, Sperelakis N. Pharmacological Actions of Calmdazolam ,a Calmodulin Antagonist, in Cardiovascular S. Cardiovascular Drug Reviews 18, 211-221. 2000.

Ref Type: Generic

(174) Ashcroft FM, Gribble FM. New windows on the mechanism of action of K(ATP) channel openers. [Review] [55 refs]. Trends in Pharmacological Sciences 2000 Nov;21(11):439-45.

(175) Lazdunski M, Lazdunski M. ATP-sensitive potassium channels: an overview. [Review] [64 refs]. J Cardiovasc Pharmacol 1994;24 Suppl 4:S1-S5.

(176) He GW, Yang CQ, He GW, Yang CQ. Comparison of nitroprusside and nitroglycerin in inhibition of angiotensin II and other vasoconstrictor-mediated contraction in human coronary bypass conduits. British Journal of Clinical Pharmacology 1997 Oct;44(4):361-7.

(177) van Son JA, Smedts F, Vincent JG, van Lier HJ, Kubat K. Comparative anatomic studies of various arterial conduits for myocardial revascularization. J Thorac Cardiovasc Surg 1990 Apr;99(4):703-7.

(178) Stojnic N, Gojkovic-Bukarica L, Peric M, Grbovic L, Lesic A, Bumbasirevic M, et al. Potassium channel opener pinacidil induces relaxation of the isolated human radial artery. Journal of Pharmacological Sciences 2007 Jun;104(2):122-9.

(179) Somlyo AP, Somlyo AV. Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. [Review] [94 refs]. Journal of Physiology 2000 Jan 15;522:t-85.

(180) Sato M, Tani E, Fujikawa H, Kaibuchi K, Sato M, Tani E, et al. Involvement of Rho-kinase-mediated phosphorylation of myosin light chain in enhancement of cerebral vasospasm.[see comment]. Circ Res 2000 Aug 4;87(3):195-200.

(181) Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, et al. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase)[see comment]. Science 1996 Jul 12;273(5272):245-8.

(182) Kim I, Leinweber BD, Morgalla M, Butler WE, Seto M, Sasaki Y, et al. Thin and thick filament regulation of contractility in experimental cerebral vasospasm. Neurosurgery 2000 Feb;46(2):440-6.

(183) Shimokawa H, Seto M, Katsumata N, Amano M, Kozai T, Yamawaki T, et al. Rho-kinase-mediated pathway induces enhanced myosin light chain phosphorylations in a swine model of coronary artery spasm. Cardiovascular Research 1999 Sep;43(4):1029-39.

(184) Kirschenlohr HL, Metcalfe JC, Weissberg PL, Grainger DJ. Proliferation of human aortic vascular smooth muscle cells in culture is modulated by active TGF beta. Cardiovascular Research 1995 Jun;29(6):848-55.

(185) Mulvany MJ, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ Res 1977 Jul;41(1):19-26.

(186) Cohen RA, Shepherd JT, Vanhoutte PM. Inhibitory role of the endothelium in the response of isolated coronary arteries to platelets. Science 1983 Jul 15;221(4607):273-4.

(187) He GW, Fan L, Furnary A, Yang Q. A new antispastic solution for arterial grafting: nicardipine and nitroglycerin cocktail in preparation of internal thoracic and radial arteries for coronary surgery. J Thorac Cardiovasc Surg 680;136(3):673-80.

(188) He GW, Buxton B, Rosenfeldt FL, Wilson AC, Angus JA. Weak beta-adrenoceptor-mediated relaxation in the human internal mammary artery. J Thorac Cardiovasc Surg 1989 Feb;97(2):259-66.

(189) He GW, Rosenfeldt FL, Buxton BF, Angus JA. Reactivity of human isolated internal mammary artery to constrictor and dilator agents. Implications for treatment of internal mammary artery spasm. Circulation 1989 Sep;80(3:Pt 1):t-50.

(190) He GW, Rosenfeldt FL, Angus JA. Pharmacological relaxation of the saphenous vein during harvesting for coronary artery bypass grafting. Ann Thorac Surg 1993 May;55(5):1210-7.

(191) He GW, Buxton BF, Rosenfeldt FL, Angus JA, Tatoulis J. Pharmacologic dilatation of the internal mammary artery during coronary bypass grafting. J Thorac Cardiovasc Surg 1994 Jun;107(6):1440-4.

(192) He GW. Verapamil plus nitroglycerin solution maximally preserves endothelial function of the radial artery: comparison with papaverine solution. J Thorac Cardiovasc Surg 1998 Jun;115(6):1321-7.

(193) Taggart DP, Dipp M, Mussa S, Nye PC. Phenoxybenzamine prevents spasm in radial artery conduits for coronary artery bypass grafting. J Thorac Cardiovasc Surg 2000 Oct;120(4):815-7.

(194) Seidel CL, Lewis RM, Bowers R, Bukoski RD, Kim HS, Allen JC, et al. Adaptation of canine saphenous veins to grafting. Correlation of contractility and contractile protein content. Circ Res 1984 Jul;55(1):102-9.

(195) British National Formulary. 2005, p. 95.

(196) Iaco AL, Teodori G, Di Giammarco G, Di Mauro M, Storto L, Mazzei V, et al. Radial artery for myocardial revascularization: long-term clinical and angiographic results. Annals of Thoracic Surgery 2001 Aug;72(2):464-8.

(197) Reardon MJ, Conklin LD, Reardon PR, Baldwin JC. Coronary artery bypass conduits: review of current status. [Review] [74 refs]. J Cardiovasc Surg (Torino) 1997 Jun;38(3):201-9.

(198) Chanda J, Canver CC. Reversal of preexisting vasospasm in coronary artery conduits. Ann Thorac Surg 2001 Aug;72(2):476-80.

(199) Raiteri M, Levi G. A reinterpretation of tyramine sympathomimetic effect and tachyphylaxis. Journal of Neuroscience Research 1986;16(2):439-41.

(200) Brunton LL. Goodman and Gillman'sThe pharmacological basis of therapy. 2006.

(201) BHAGAT B. Tyramine-induced depletion of cardiac catecholamines and the effects of cocaine and bretylium. Archives Internationales de Pharmacodynamie et de Therapie 1964 Jan 1;147:26-35.

(202) IVERSEN LL, WHITBY LC. The subcellular distributijon of catecholamines in normal and tyramine-depleted mouse hearts. Biochemical Pharmacology 1963 Jun;12:582-4.

(203) von EU, LISHAJKO F. Release of noradrenaline from adrenergic transmitter granules by tyramine. Experientia 1960 Aug 15;16:376-7.

(204) Neff NH, Tozer TN, Hammer W, Brodie BB. Kinetics of release of norepinephrine by tyramine. Life Sciences 1965 Oct;4(19):1869-75.

(205) Rapoport RM, Takimoto GS, Cho AK. Compartmental analysis of tyramine-induced norepinephrine depletion. Pharmacology 1981;22(4):235-42.

(206) Langer SZ, Shepperson NB, Massingham R. Preferential noradrenergic innervation of alpha-adrenergic receptors in vascular smooth muscle. Hypertension 1981 May;3(3 Pt 2):I112-I118.

(207) Langer SZ, Hicks PE. Alpha-adrenoreceptor subtypes in blood vessels: physiology and pharmacology. [Review] [75 refs]. J Cardiovasc Pharmacol 1984;6 Suppl 4:S547-S558.

(208) Medgett IC, Hicks PE, Langer SZ. Smooth muscle alpha-2 adrenoceptors mediate vasoconstrictor responses to exogenous norepinephrine and to sympathetic stimulation to a greater extent in spontaneously hypertensive than in Wistar Kyoto rat tail arteries. J Pharmacol Exp Ther 1984 Oct;231(1):159-65.

(209) Parkinson NA, Thom SM, Hughes AD, Sever PS, Mulvany MJ, Nielsen H. Neurally evoked responses of human isolated resistance arteries are mediated by both alpha 1- and alpha 2-adrenoceptors. Br J Pharmacol 1992 Jul;106(3):568-73.

(210) Makhoul RG, Davis WS, Mikat EM, McCann RL, Hagen PO. Responsiveness of vein bypass grafts to stimulation with norepinephrine and 5-hydroxytryptamine. Journal of Vascular Surgery 1987 Jul;6(1):32-8.

(211) Okon EB, Millar MJ, Crowley CM, Bashir JG, Cook RC, Hsiang YN, et al. Effect of moderate pressure distention on the human saphenous vein vasomotor function. Ann Thorac Surg 114 May;77(1):108-14.

(212) Spray TL, Roberts WC. Changes in saphenous veins used as aortocoronary bypass grafts. Am Heart J 1977 Oct;94(4):500-16.

(213) LoGerfo FW, Quist WC, Cantelmo NL, Haudenschild CC. Integrity of vein grafts as a function of initial intimal and medial preservation. Circulation 1983 Sep;68(3:Pt 2):t-24.

(214) Dzau V. The cardiovascular continuum and renin-angiotensin-aldosterone system blockade. [Review] [74 refs]. Journal of Hypertension - Supplement 2005 Apr;23(1):S9-17.

(215) Owens GK, Schwartz SM. Vascular smooth muscle cell hypertrophy and hyperploidy in the Goldblatt hypertensive rat. Circ Res 1983 Oct;53(4):491-501.

(216) Schiffers PM, Struyker-Boudier HA, De Mey JG. Effects of angiotensin II and angiotensin converting enzyme inhibitors on contractile and growth responses in isolated carotid arteries of the rat. Basic Research in Cardiology 1991;86:Suppl-9.

(217) Turla MB, Thompson MM, Corjay MH, Owens GK. Mechanisms of angiotensin II- and arginine vasopressin-induced increases in protein synthesis and content in cultured rat aortic smooth muscle cells. Evidence for selective increases in smooth muscle isoactin expression. Circ Res 1991 Jan;68(1):288-99.

(218) Powell JS, Clozel JP, Muller RK, Kuhn H, Hefti F, Hosang M, et al. Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. Science 1989 Jul 14;245(4914):186-8.

(219) Hu ZW, Shi XY, Sakaue M, Hoffman BB. Prolonged activation of protein kinase C induces transcription and expression of the alpha 1B adrenergic receptor gene in DDT1 MF-2 cells. Journal of Biological Chemistry 1993 Feb 15;268(5):3610-5.

(220) Paul M, Ganten D. The molecular basis of cardiovascular hypertrophy: the role of the renin-angiotensin system. [Review] [47 refs]. J Cardiovasc Pharmacol 1992;19:Suppl-8.

(221) Velez DA, Morris CD, Muraki S, Budde JM, Otto RN, Zhao ZQ, et al. Brief pretreatment of radial artery conduits with phenoxybenzamine prevents vasoconstriction long term. Ann Thorac Surg 2001 Dec;72(6):1977-84.

(222) Harrison WE, Mellor AJ, Clark J, Singer DR. Vasodilator pre-treatment of human radial arteries; comparison of effects of phenoxybenzamine vs papaverine on norepinephrine-induced contraction in vitro.[see comment]. European Heart Journal 2001 Dec;22(23):2209-16.

(223) Loop FD, Lytle BW, Cosgrove DM, Stewart RW, Goormastic M, Williams GW, et al. Influence of the internal-mammary-artery graft on 10-year survival and other cardiac events. N Engl J Med 1986 Jan 2;314(1):1-6.

(224) Buxton BF, Raman JS, Ruengsakulrach P, Gordon I, Rosalion A, Bellomo R, et al. Radial artery patency and clinical outcomes: five-year interim results of a randomized trial. J Thorac Cardiovasc Surg 2003 Jun;125(6):1363-71.

(225) Ning YM, Sanchez ER. Evidence for a functional interaction between calmodulin and the glucocorticoid receptor. Biochemical & Biophysical Research Communications 1995 Mar 8;208(1):48-54.

(226) Chardigny C, Jebara VA, Acar C, Descombes JJ, Verbeuren TJ, Carpentier A, et al. Vasoreactivity of the radial artery. Comparison with the internal mammary and gastroepiploic arteries with implications for coronary artery surgery. Circulation 1993 Nov;88(5 Pt 2):II115-II127.

(227) Wickenden AD, Grimwood S, Grant TL, Todd MH. Comparison of the effects of the K(+)-channel openers cromakalim and minoxidil sulphate on vascular smooth muscle. Br J Pharmacol 1991 May;103(1):1148-52.

(228) Chester AH, Marchbank AJ, Borland JA, Yacoub MH, Taggart DP, Chester AH, et al. Comparison of the morphologic and vascular reactivity of the proximal and distal radial artery. Ann Thorac Surg 1998 Dec;66(6):1972-6.

(229) Martinez MC, Randriamboavonjy V, Ohlmann P, Komas N, Duarte J, Schneider F, et al. Involvement of protein kinase C, tyrosine kinases, and Rho kinase in Ca(2+) handling of human small arteries. American Journal of Physiology - Heart & Circulatory Physiology 2000 Sep;279(3):H1228-H1238.

(230) Hizume T, Morikawa K, Takaki A, Abe K, Sunagawa K, Amano M, et al. Sustained elevation of serum cortisol level causes sensitization of coronary vasoconstricting responses in pigs in vivo: a possible link between stress and coronary vasospasm. Circ Res 2006 Sep 29;99(7):767-75.

(231) Hall A, Hall A. Rho GTPases and the actin cytoskeleton. [Review] [50 refs]. Science 1998 Jan 23;279(5350):509-14.

(232) Horwitz AR, Parsons JT, Horwitz AR, Parsons JT. Cell migration--movin' on.[comment]. Science 1999 Nov 5;286(5442):1102-3.

(233) Narumiya S, Narumiya S. The small GTPase Rho: cellular functions and signal transduction. [Review] [117 refs]. Journal of Biochemistry 1996 Aug;120(2):215-28.

(234) Funakoshi Y, Ichiki T, Shimokawa H, Egashira K, Takeda K, Kaibuchi K, et al. Rho-kinase mediates angiotensin II-induced monocyte chemoattractant protein-1 expression in rat vascular smooth muscle cells. Hypertension 2001 Jul;38(1):100-4.

(235) van Nieuw Amerongen GP, van DS, Vermeer MA, Collard JG, van Hinsbergh VW. Activation of RhoA by thrombin in endothelial hyperpermeability: role of Rho kinase and protein tyrosine kinases. Circ Res 2000 Aug 18;87(4):335-40.

(236) Sawada N, Itoh H, Ueyama K, Yamashita J, Doi K, Chun TH, et al. Inhibition of rho-associated kinase results in suppression of neointimal formation of balloon-injured arteries. Circulation 2000 May 2;101(17):2030-3.

(237) Davies SP, Reddy H, Caivano M, Cohen P, Davies SP, Reddy H, et al. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochemical Journal 2000 Oct 1;351(Pt 1):95-105.

(238) Arai T, Takeyama N, Tanaka T, Arai T, Takeyama N, Tanaka T. Glutathione monoethyl ester and inhibition of the oxyhemoglobin-induced increase in cytosolic calcium in cultured smooth-muscle cells.[see comment]. Journal of Neurosurgery 1999 Mar;90(3):527-32.

(239) Vollrath BA, Weir BK, Macdonald RL, Cook DA. Intracellular mechanisms involved in the responses of cerebrovascular smooth-muscle cells to hemoglobin. Journal of Neurosurgery 1994 Feb;80(2):261-8.

(240) Masumoto A, Mohri M, Shimokawa H, Urakami L, Usui M, Takeshita A, et al. Suppression of coronary artery spasm by the Rho-kinase inhibitor fasudil in patients with vasospastic angina.[see comment]. Circulation 2002 Apr 2;105(13):1545-7.

(241) Fu X, Gong MC, Jia T, Somlyo AV, Somlyo AP. The effects of the Rho-kinase inhibitor Y-27632 on arachidonic acid-, GTPgammaS-, and phorbol ester-induced Ca2+-sensitization of smooth muscle. FEBS Letters 1998 Nov 27;440(1-2):183-7.

(242) Iizuka K, Yoshii A, Samizo K, Tsukagoshi H, Ishizuka T, Dobashi K, et al. A major role for the rho-associated coiled coil forming protein kinase in G-protein-mediated Ca2+ sensitization through inhibition of myosin phosphatase in rabbit trachea. Br J Pharmacol 1999 Oct;128(4):925-33.

(243) Etter EF, Eto M, Wardle RL, Brautigan DL, Murphy RA, Etter EF, et al. Activation of myosin light chain phosphatase in intact arterial smooth muscle during nitric oxide-induced relaxation. Journal of Biological Chemistry 2001 Sep 14;276(37):34681-5.

(244) Takemoto M, Sun J, Hiroki J, Shimokawa H, Liao JK, Takemoto M, et al. Rho-kinase mediates hypoxia-induced downregulation of endothelial nitric oxide synthase. Circulation 2002 Jul 2;106(1):57-62.

(245) Fulton D, Gratton JP, Sessa WC, Fulton D, Gratton JP, Sessa WC. Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough?. [Review] [52 refs]. J Pharmacol Exp Ther 2001 Dec;299(3):818-24.

(246) Budzyn K, Marley PD, Sobey CG, Budzyn K, Marley PD, Sobey CG. Targeting Rho and Rho-kinase in the treatment of cardiovascular disease. [Review] [61 refs]. Trends in Pharmacological Sciences 2006 Feb;27(2):97-104.

(247) Acar C, Buxton B, Eizenberg N. Radial artery. In: Buxton B, Frazier OH, Westaby S, editors. Ischaemic Heart Disease Surgical Management. London: Mosby International Limited; 2004, p. 151-7.

(248) Barner HB, Sundt TM, III, Bailey M, Zang Y. Midterm results of complete arterial revascularization in more than 1,000 patients using an internal thoracic artery/radial artery T graft. Annals of Surgery 2001 Oct;234(4):447-52.

(249) Beghi C, Nicolini F, Budillon AM, Borrello B, Ballore L, Reverberi C, et al. Midterm clinical results in myocardial revascularization using the radial artery. Chest 2002 Dec;122(6):2075-9.

(250) Calafiore AM, Di Mauro M, D'Alessandro S, Teodori G, Vitolla G, Contini M, et al. Revascularization of the lateral wall: long-term angiographic and clinical results of radial artery versus right internal thoracic artery grafting. Journal of Thoracic & Cardiovascular Surgery 2002 Feb;123(2):225-31.

ELSEVIER

European Journal of Cardio-thoracic Surgery 34 (2008) 839–844

EUROPEAN JOURNAL OF CARDIO-THORACIC SURGERY

www.elsevier.com/locate/ejcts

Attenuation of receptor-dependent and -independent vasoconstriction in the human radial artery☆

Michael J. Shackcloth[a], Alan R. Conant[a,*], Joyce Thekkudan[a], Sanjay Ghotkar[a], Alec W.M. Simpson[b], Walid C. Dihmis[a]

[a] The Cardiothoracic Centre, Thomas Drive, Liverpool, UK
[b] Department of Human Anatomy & Cell Biology, University of Liverpool, UK

Received 10 January 2008; received in revised form 9 June 2008; accepted 11 June 2008; Available online 3 August 2008

Abstract

Background: Vasodilator strategies used to treat bypass grafts in the operating theatre, such as nitrates, phosphodiesterase inhibitors and calcium channel antagonists have a broad but short-lived effect against a variety of vasoconstrictor stimuli. Treatments that react irreversibly with proteins modulating vasoconstriction have the advantage that their effects can last well into the postoperative period. In addition systemic effects are avoided as the treatment is localised to the treated graft. This study investigated the use of two clinically applied drugs; fluphenazine (SKF7171A, HCl), an irreversible calmodulin antagonist and minoxidil sulphate, an irreversible potassium channel opener. Treatments were tested against receptor and non-receptor-mediated contraction in the human radial artery. **Method:** Isometric tension was measured in response to angiotensin II, KCl and vasopressin in 108 radial artery rings (taken from 31 patients undergoing coronary artery bypass grafting). Control responses were compared with rings pretreated with fluphenazine or minoxidil sulphate. Vasopressin responses were also compared in the presence of glyceryl trinitrate or the reversible Rho kinase inhibitor Y27632. **Results:** Fluphenazine pretreatment significantly suppressed vasoconstriction to all agonists tested. Maximal responses to angiotensin II, vasopressin and KCl were reduced by 42 ± 19%, 35 ± 8% and 48 ± 15% respectively, without any measurable effect on the EC_{50}. Minoxidil sulphate showed no discernable effect. Vasopressin-induced contraction was also reduced by high levels of glyceryl trinitrate (220 μM; 50 μg/ml) or 10 μM Y27632. **Conclusions:** The irreversible calmodulin antagonist fluphenazine has potential to be developed as an inhibitor of contraction in arterial graft vessels. The involvement of Rho kinase indicates that other vasoconstrictors and surgical stress can sensitize radial artery to vasopressin-induced contraction. Strategies targeting this pathway also have future potential.

Keywords: Arteries; Artery biochemistry/pharmacology; CABG; Arterial grafts; CABG; Pharmacology/physiology; Cell signaling; Receptors

1. Introduction

In coronary artery bypass graft surgery (CABG) superior patient survival rates and increased freedom from major adverse cardiac events have been obtained in patients receiving left internal thoracic artery (ITA) grafts as opposed to saphenous vein grafts [1]. These results have encouraged surgeons towards a complete arterial revascularisation strategy. Promising patency rates obtained with the radial artery have now also established this graft as a good alternative to the right ITA [2]. The radial artery is a comparatively reactive graft and vasodilator strategies are topically applied by many surgeons in theatre and intravenously in the postoperative period to ameliorate vasospasm, which is believed to account for a proportion of early graft failures [2,3]. Vasodilator therapies include combinations of nitrates, phosphodiesterase inhibitors or calcium channel antagonists, and the recently introduced irreversible α adrenoceptor antagonist phenoxybenzamine [4,2,5]. The beneficial effects of this agent have been shown in a recent prospective study. Patients receiving phenoxybenzamine-treated grafts had a lower level of perioperative myocardial injury and a reduced incidence of adverse cardiac events when compared to a verapamil/glyceryl trinitrate treated group [5]. The strategy of using an irreversible inhibitor is very attractive, since it means that pharmacologically effective concentrations can be applied selectively to the graft and the effect maintained in the perioperative period without systemic complications.

Phenoxybenzamine has demonstrated the potential benefits of irreversible antagonists in CABG as treatment completely abolishes noradrenaline-induced contraction in

☆ Supported by the British Heart Foundation and the Merseybeat Appeal (The Cardiothoracic Centre, Liverpool, UK). MS was supported by a British Heart Foundation Junior Research Fellowship (FS/03/057) during this project.

* Corresponding author. Current address: Physiology Dept, University of Liverpool, Crown St, Liverpool L69 3BX, UK. Tel.: +44 151 7945510; fax: +44 151 7945517.
E-mail address: conant@liv.ac.uk (A.R. Conant).

1010-7940/$ – see front matter
doi:10.1016/j.ejcts.2008.06.016

radial artery for up to 16 h in vivo; unfortunately however the beneficial effects are limited to catecholamine-mediated vasoconstriction [6–8]. Vasoconstrictors other than catecholamines, released into the plasma in the immediate postoperative period [9] can still reduce flow through the treated graft [3]. Our aim was to test the potential for clinical application of other irreversible antagonists, with a broader range of activity and the potential to inhibit both receptor and non-receptor-mediated contraction in the radial artery. We tested two drugs used currently in other clinical settings, fluphenazine and minoxidil sulphate. Fluphenazine is a cell permeable, irreversible antagonist of calmodulin [10], the cellular protein that translates an increase in intracellular calcium into contraction. Minoxidil sulphate binds irreversibly to the vascular ATP-sensitive K^+ channel (K_{ATP} channel) and is used as an antihypertensive agent [11].

Radial artery produces stronger vasoconstriction than ITA to virtually all vasoconstrictors studied [12–15]; however vasoconstriction is equivalent when normalised to account for the different vessel diameters in most cases. However, normalised responses to both angiotensin II and vasopressin in the radial artery are stronger and more sensitive than in the ITA, and occur irrespective of the presence of endothelium [12,14,15]. In addition, vasopressin-induced contraction in the radial artery is comparatively resistant to milrinone and glyceryl trinitrate, two of the most commonly used vasodilator strategies [15]. The inhibition of the vasopressin-induced contraction in radial artery grafts would be particularly advantageous for surgeons, since they may wish to use vasopressin to treat hypotension in the postoperative period [16]. In this study we investigated the ability of fluphenazine and minoxidil to inhibit contraction to vasopressin, angiotensin II and high potassium. Vasopressin data were compared with glyceryl trinitrate and with a Rho kinase inhibitor Y27632, as the inhibition of Rho kinase has been shown to reduce coronary artery vasospasm [17]. The potential of these treatments, particularly fluphenazine, as graft treatments in bypass surgery is discussed.

2. Material and methods

2.1. Sample preparation

Samples of radial artery were obtained from 31 patients undergoing CABG at the Cardiothoracic Centre, Liverpool NHS Trust, UK. The clinical characteristics of these patients are given in Table 1. The study was approved by the Liverpool Research ethics committee and informed consent was obtained from each patient. The radial artery was harvested with surrounding fat and the two satellite veins. Depending on the practice of the surgeons concerned, radial arteries were treated in the theatre with either 1.6 mM papaverine (Martindale Pharmaceuticals, Romford, UK) or 6 mM phenoxybenzamine (Goldshield Pharmaceuticals Ltd., Croydon, UK) in a solution of the patients' whole blood for 30 min. Arterial sections surplus to surgical requirements were collected from theatre into Dulbecco's Modified Eagle's Media (Invitrogen, UK) on ice and immediately transported to the research laboratories.

Table 1
Clinical characteristics of patients

Total no of patients	31
Mean age (years)	63
Sex ratio (men/women)	23/8
Risk factors (*n*)	
Smoking	6
Arterial hypertension	15
Diabetes mellitus	6
Preoperative treatment (*n*)	
β-Blockers	22
Calcium-channel blockers	14
Nitrates	15
K+ openers	9
ACE inhibitors	11

2.2. Organ bath contraction studies

Radial artery sections were cut into 2–3 mm rings giving 108 rings in total. Arterial rings were suspended in thermostatically controlled organ chambers at 37 °C, filled with 25 ml Krebs–Henseleit buffer composed of 118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM D-glucose, 1 mM $CaCl_2$, gassed with carbogen (95% O_2/5% CO_2). Rings were mounted between wire stirrups connected to a force transducer and changes in isometric force recorded on a PowerLab 16SP data recording system, connected to an Octal ML119 bridge amplifier (ADInstruments Ltd., Chalgrove, Oxfordshire, UK). Prior to stimulation radial artery rings underwent a pretensioning protocol based on that described by He and Yang [14]. Arterial rings were set at a pretension equivalent to 100 mmHg, calculated from the internal circumference and the wall tension derived from the Laplace relationship. The tension was readjusted to the equivalent of 100 mmHg every 20 min over a 40-min period. All rings were then partially relaxed and left resting at approximately 90% of their maximal circumference for 20 min prior to study. To test for functional contractility, arterial rings were first stimulated with 90 mM KCl and all other responses, including later KCl responses, expressed as % of this response. Following surgical preparation and our contraction protocol, endothelial function, as evidenced by a vasodilatory response to 10 μM acetylcholine in rings precontracted with 30 mM KCl, was found to be negligible. Responses were terminated by washing with three complete changes of media. Rings pretreated with fluphenazine-*N*-2-chloroethane, diHCl (SKF7171A, HCl; Merck Chemicals Ltd., Nottingham, UK) were incubated in the presence of the drug for 40 min at 100 μM, 10-fold greater than the IC_{50} concentration for calmodulin [10]. Rings treated with minoxidil sulphate (Merck Chemicals Ltd.) were incubated for 30 min at 10 μM, a concentration reported to completely reverse noradrenaline-induced contraction in vascular smooth muscle [18]. Following incubation with fluphenazine or minoxidil sulphate the drug was removed by three complete changes of media. Responses to angiotensin II (Sigma, Poole, UK) were recorded within 20 min of drug washout and responses to Arg-vasopressin (Sigma) at either 30 or 120 min. Responses in treated rings

were normalised to control rings stimulated in parallel and pretreated with vehicle alone (dimethylsulphoxide; DMSO). Y27632 (Merck Chemicals Ltd.) or glyceryl trinitrate (DBL, Warwick, UK) were added to the organ chamber 5 min prior to and during agonist addition.

2.3. Data analysis

Data are presented as mean ± standard deviation of the mean (SD), where *n* refers to the number of independent samples tested. The threshold vasopressin concentration was defined as the concentration at which tension was significantly raised above baseline tension. Data were tested for normal distribution and appropriate comparisons were undertaken as the analysis dictated using a one-way ANOVA and a Bonferroni correction for multiple comparisons. All analyses were carried out using the program Arcus QuickStat Biomedical using a p value of 0.05 (Hearne Scientific Software, Dublin, Eire).

3. Results

The internal diameter at a pressure of 100 mmHg was 3.8 ± 0.9 mm. To test for functional vasoconstriction, sections of human radial artery were stimulated with 90 mM KCl that raised tension from a mean basal value of 1.93 ± 0.92 g (n = 31) to 7.70 ± 3.66 g. Data were collected from distal arterial sections treated with phenoxybenzamine in theatre (6.26 ± 2.18 g; n = 17) and proximal sections treated with papaverine (9.72 ± 4.49 g; n = 14). As previously reported by other investigators [19], proximal sections gave significantly higher KCl-induced contraction than distal ($p < 0.05$). All data were therefore expressed as % of this initial KCl response.

3.1. Vasoconstrictor responses in radial artery

Robust concentration dependent contraction was observed to vasopressin and angiotensin II giving mean maximal responses of 109 ± 10% KCl (n = 13; 95% confidence interval 104–115) and 87 ± 20% KCl (n = 14; 95% confidence interval 104–79) respectively (Fig. 1a and b). When expressed as % KCl, maximal responses to vasopressin and angiotensin II were not significantly different from proximal and distal sections. Maximal responses to vasopressin were also reproducible when arterial rings were compared from the same sample giving an average standard deviation 6.3 ± 4.0% for the response (n = 5). Maximal responses to angiotensin II were more variable giving an average standard deviation 15 ± 8% for the response (n = 3). Mean EC_{50} values ($-\log_{10} M$) of 9.16 ± 0.39 (95% confidence interval 8.94–9.39) and 8.33 ± 0.52 (95% confidence interval 7.93–8.73) were obtained for vasopressin and angiotensin II respectively. Whilst maximal responses to both KCl and angiotensin II returned to baseline within 10 min following agonist washout, vasopressin-induced contraction was completely reversed only by glyceryl trinitrate. A mean EC_{50} value for the reversal of maximal vasopressin-induced contraction with glyceryl trinitrate was 4.05 ± 1.02 ($-\log_{10} M$; n = 6).

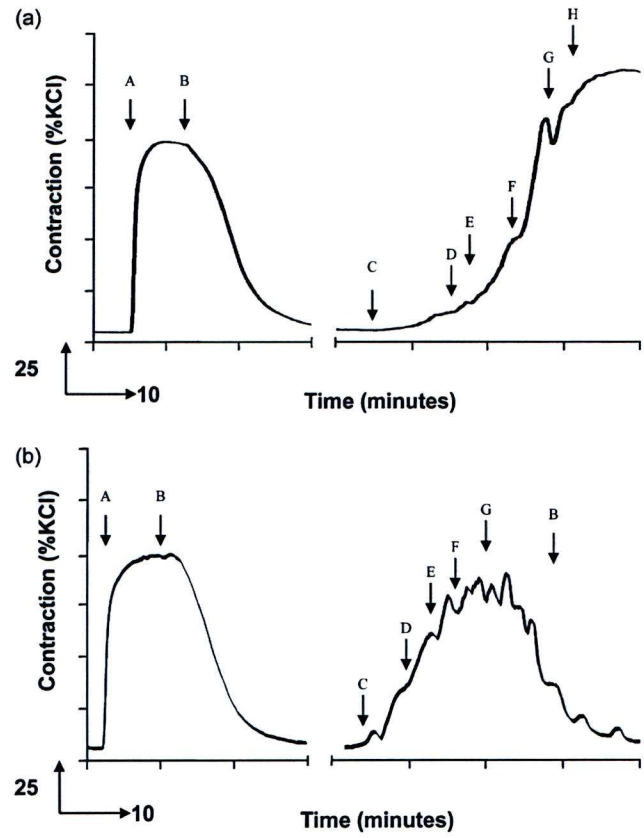

Fig. 1. Representative traces from individual rings of radial artery showing standard responses to increasing concentrations of either vasopressin or angiotensin II. Responses are shown in relation to an initial response to 90 mM KCl (*A*). Following washout (*B*) of the KCl response with two complete changes of media, rings were stimulated with increasing concentrations of either vasopressin (a) where C = 0.1, D = 0.3, E = 1, F = 10, G = 30 and H = 100 nM vasopressin) or angiotensin II (b) where C = 1, D = 3, E = 10, F = 30 and G = 100 nM angiotensin II).

3.2. Effect of treatments against vasopressin-induced contraction

Pretreatment with fluphenazine consistently reduced the maximal vasopressin-induced contraction (Fig. 2a; Table 2). When inhibition was measured at 30 min (n = 3) and 140 min (n = 3) after the drug had been washed out there was no loss of effect. In addition, inhibition was identical in vessels treated with phenoxybenzamine in theatre (n = 4) compared to papaverine (n = 3). Arterial rings pretreated with minoxidil failed to show any measurable inhibition (Fig. 2a; Table 2). The effects of the Rho kinase inhibitor Y27632 were also tested against vasopressin-induced contraction and compared with glyceryl trinitrate (Fig. 2b). The addition of 10 μM Y27632 to the organ chamber reduced the basal tension by 33 ± 15% (n = 5) and resulted in a significant reduction in the maximal vasopressin-induced contraction (Fig. 2b). Y27632 did not demonstrate any measurable effect on the EC_{50} of the response for vasopressin and the degree of inhibition of contraction was comparable to that produced by 220 μM glyceryl trinitrate (Table 2). One consequence of the inhibitory effects of Y27632 and glyceryl trinitrate was to increase the threshold vasopressin concentration at which a significant contraction could be measured from 0.1 to 1.0 nM vasopressin.

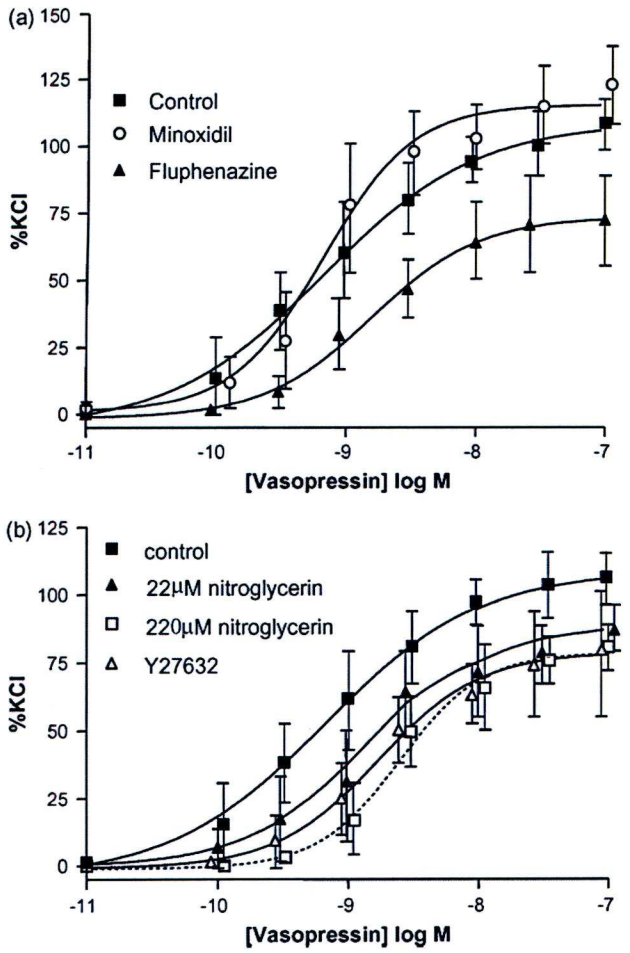

Fig. 2. Effect of treatments against vasopressin-mediated vasoconstriction. Cumulative concentration response curves were obtained to vasopressin following pretreatment (a) with vehicle alone (control; $n = 12$), 100 μM fluphenazine ($n = 6$) or 10 μM minoxidil sulphate ($n = 6$) or in the presence of (b) 10 μM Y27632 ($n = 5$) and 22 μM ($n = 5$) and 220 μM ($n = 5$) glyceryl trinitrate, where n refers to the number of independent samples tested. Average responses ± SD, are shown relative to a contractile response to 90 mM KCl applied prior to treatment.

3.3. Effect of minoxidil sulphate and fluphenazine pretreatment against angiotensin II and KCl-induced vasoconstriction

Maximal responses to angiotensin II were also significantly reduced by pretreatment with fluphenazine but not minoxidil when compared to controls (Table 3). No measurable change in EC_{50} values for vasopressin was observed with either treatment. In addition, following fluphenazine treatment, responses to 90 mM KCl were reduced by 48 ± 15%, when compared to the initial KCl response. In control rings, KCl responses were maintained, giving average values 107 ± 21% of the initial contraction ($n = 8$). Pretreatment with minoxidil sulphate showed no measurable effect against KCl-induced contraction giving an average response 112 ± 17% ($n = 6$) of the initial contraction.

Table 3
Comparison of maximal responses and affinity of response to angiotensin II in human radial artery pretreated with minoxidil sulphate or fluphenazine

	Minoxidil	Fluphenazine
Max response (% KCl)	80 ± 28	54 ± 20[a]
Confidence intervals (95%)	107–55	75–32
n	7	6
EC_{50} (−log M)	8.28 ± 0.34	8.13 ± 0.86
Confidence intervals (95%)	7.86–8.69	6.76–9.51

Data are expressed as % response to 90 mM KCl, where control responses to angiotensin II were 87 ± 20% with an EC_{50} of 8.33 ± 0.52 ($n = 14$). n refers to the number of independent samples tested.

[a] Denotes significant differences ($p < 0.05$) between treatment groups and controls by ANOVA using the Bonferroni correction for multiple comparisons.

4. Discussion

The major finding of this study was that pretreatment of radial artery grafts with the calmodulin inhibitor, fluphenazine (SKF7171A), significantly attenuated receptor-mediated contraction evoked by angiotensin II and vasopressin, and depolarisation-induced contraction by high potassium. Calmodulin is the cellular protein, which integrates the calcium response and activates the myosin light chain kinase responsible for contraction. Since an increase in smooth muscle calcium is the primary initiating stimulus for contraction, this makes calmodulin an excellent target for inhibiting contraction evoked by a wide range of agonists. The true incidence of vasospasm in the human radial artery is difficult to determine as many cases may go undetected [2,3]. Reports of perioperative ischaemia in patients with radial artery bypass grafts [20] and the observed reduction in postoperative markers of myocardial infarction in patients receiving radial artery grafts treated with phenoxybenzamine as compared with those treated with verapamil/glyceryl trinitrate [5], would support the need for effective, postoperative vasodilator therapy.

Table 2
Maximal responses and affinity of response to vasopressin in radial artery for treatment groups compared with vehicle treated controls

	Minoxidil	Fluphenazine	Y27632	Glyceryl trinitrate (22 μM)	Glyceryl trinitrate (220 μM)
Max response (% KCl)	119 ± 16	72 ± 12[a]	94 ± 13[a]	99 ± 12	81 ± 11
Confidence intervals (95%)	134–105	81–63	110–79	114–83	95–68
n	7	7	5	5	5
EC_{50} (−log M)	9.13 ± 0.32	8.90 ± 0.35	8.81 ± 0.33	8.78 ± 0.43	8.56 ± 0.38[a]
Confidence intervals (95%)	8.84–9.43	8.58–9.22	8.41–9.22	8.25–9.31	8.09–9.03

Data are expressed as % response to 90 mM KCl, where control responses to vasopressin were 108 ± 11% with an EC_{50} of 9.16 ± 0.39 ($n = 13$). n refers to the number of independent samples tested.

[a] Denotes significant differences ($p < 0.05$) when treatment groups were compared with controls by ANOVA using the Bonferroni correction for multiple comparisons.

The radial artery is a muscular artery with a larger medial layer and a higher concentration of myocytes, when compared with the ITA, and this is believed to underlie its reactive nature [21]. In the immediate postoperative period when circulating vasoconstrictor levels are still elevated [9] systemic vasodilators are used by many surgeons to minimise the potential for vasospasm. Strategies include glyceryl trinitrate used either alone or in combination with calcium channel antagonists and the phosphodiesterase inhibitor milrinone [2–4]. A key observation is that fluphenazine inhibited vasopressin-induced contraction by a similar extent to that of the maximal dose of glyceryl trinitrate used. Since many of the samples used in this study were already treated with the irreversible α adrenergic antagonist phenoxybenzamine the effects of fluphenazine are additional to those of phenoxybenzamine. Phenoxybenzamine is now used by many surgeons and our results suggest that fluphenazine can be used alongside phenoxybenzamine, to provide inhibition to a broader spectrum of stimuli.

Potent vasodilatation with nicorandil, a combined potassium channel opener and nitric oxide donor, has been reported against endothelin-mediated contraction in radial artery [22]. Pinacidil, which shares a common binding site with minoxidil sulphate and nicorandil on the K_{ATP} channel, has also demonstrated its ability to reverse phenylephrine-induced contraction in human radial artery [23]. Therefore the activation of K_{ATP} channels would be expected to reverse membrane depolarisation, inhibit calcium influx across the plasma membrane and relax the precontracted radial artery [11]. We found minoxidil sulphate to be ineffective at inhibiting contractions in the radial artery. This occurred irrespective of the use of potassium channel blockers in the preoperative medication. Interestingly, minoxidil sulphate demonstrates a high degree of tissue and species selectivity. For example, minoxidil sulphate is a potent vasodilator in rat aorta but not in rabbit aorta or rat mesenteric artery, whereas other K_{ATP} channel openers were equally effective in all tissues [18]. The lack of effect measured in human radial artery in this study indicates that minoxidil sulphate has poor activity in this vessel also.

The effect of the reversible Rho kinase inhibitor Y27632 was also investigated. Rho kinase controls the activation state of the myosin light chain kinase responsible for the phosphorylation of myosin. Activation of Rho kinase leads to vasoconstriction at basal intracellular calcium levels in smooth muscle [17]. This study demonstrated that Y27632 reduced the maximal vasopressin-induced contraction. Rho kinase is activated by serotonin, endothelin 1 and thromboxane A_2 [17] and by the stress hormone cortisol [24]. Therefore the involvement of the Rho kinase pathway in vasopressin-induced contraction in the radial artery indicates that both surgical stress and elevated levels of other vasoconstrictors can increase the sensitivity of the graft to vasopressin. This may be pertinent to the management of postoperative contraction in radial artery grafts or the use of vasopressin in the management of hypotension following CABG [16], particularly as vasopressin-induced contraction in radial artery is refractory to two of the most commonly used vasodilator strategies [15].

4.1. Limitations of this study

This study demonstrated significant inhibition of both receptor and non-receptor-mediated vasoconstriction in radial artery with fluphenazine. Although effects were only measured up to 2 h after treatment they would be expected to be similar in duration to phenoxybenzamine, since irreversible interactions of this type will last until the smooth muscle cells of the vessel wall replace the inactivated protein [7,8]. Inhibition with fluphenazine should therefore last well into the postoperative period [8]; however it would be useful to verify this in vivo. The animal model used to confirm the duration of action of phenoxybenzamine would suit this purpose [8]. Using this model it was demonstrated that the effects of phenoxybenzamine lasted for 16 h in vivo and we would expect a similar duration of action for fluphenazine. In addition, calmodulin inhibition could also affect the generation of endothelium-derived vasodilators. However the generation of vasodilators by the endothelium is not necessarily calmodulin-mediated [25] and it is unclear how calmodulin inhibition would affect endothelium-dependent vasodilatation in the radial artery. Interestingly, the presence or absence of endothelium has been shown not to influence vasopressin-induced vasoconstriction [15]; however, the effects of fluphenazine treatment on endothelium-mediated vasodilatation should also be considered.

4.2. Conclusion

Fluphenazine has potential to be developed as a useful treatment in graft vessels and demonstrates long-lasting effects against vasoconstriction induced by several unrelated agonists. Further studies are required to investigate the duration and breadth of effect of fluphenazine against a broader range of vasoconstrictors in radial artery. The development of clinically useful Rho kinase inhibitors may also provide a future therapeutic opportunity for the management of arterial contraction in the postoperative period, especially if Rho kinase inhibitors can be developed to give long-lasting protection [17].

References

[1] Loop FD, Lytle BW, Cosgrove DM, Stewart RW, Goormastic M, Williams GW, Golding LAR, Gill CC, Taylor PC, Sheldon WC, Proudfit WL. Influence of the internal mammary artery graft on 10-year survival and other cardiac events. New Eng J Med 1986;314:1–6.

[2] Desai ND, Fremes SE. Radial artery conduit for coronary revascularization: as good as an internal thoracic artery? Curr Opin Cardiol 2007;22:534–40.

[3] Rosenfeldt FL, He GW, Buxton BF, Angus JA. Pharmacology of coronary artery bypass grafts. Ann Thorac Surg 1999;67:878–88.

[4] Buxton BF, Raman JS, Ruengsakulrach P, Gordon I, Rosalion A, Bellomo R, Horrigan M, Hare DL. Radial artery patency and clinical outcomes: five-year interim results of a randomized trial. J Thorac Cardiovasc Surg 2003;125:1363–71.

[5] Kulik A, Rubens FD, Gunning D, Bourke ME, Mesana TG, Ruel M. Radial artery graft treatment with phenoxybenzamine is clinically safe and may reduce perioperative myocardial injury. Ann Thorac Surg 2007;83:502–9.

[6] Conant AR, Shackcloth MS, Oo AY, Chester MR, Simpson AWM, Dihmis WC. Phenoxybenzamine treatment is insufficient to prevent spasm in the radial artery – the effect of other vasodilators. J Thorac Cardiovasc Surg 2003;126:448–54.

[7] Mussa S, Guzik TJ, Black E, Dipp MA, Channon KM, Taggart DP. Comparative efficacies and durations of action of phenoxybenzamine, verapamil/nitroglycerin solution, and papaverine as topical antispasmodics for radial artery coronary bypass grafting. J Thorac Cardiovasc Surg 2003;126:1798–805.
[8] Mussa S, Prior T, Alp N, Wood K, Channon KM, Taggart DP. Duration of action of antispasmodic agents: novel use of a mouse model as an in vivo pharmacological assay. Eur J Cardiothorac Surg 2004;26:988–94.
[9] Downing SW, Edmunds LH. Release of vasoactive substances during cardiopulmonary bypass. Ann Thorac Surg 1992;54:1236–43.
[10] Hait WN, Glazer L, Kaiser C, Cross J, Kennedy KA. Pharmacological properties of fluphenazine-mustard, an irreversible calmodulin antagonist. Mol Pharmacol 1987;32:404–9.
[11] Ashcroft FM, Gribble FM. New windows on the mechanism of action of K-ATP channel openers. Trend Pharmacol Sci 2000;21:439–45.
[12] Borland JAA, Chester AH, Rooker SJ, Wharton J, Davie N, Amrani M, Yacoub MH. Expression and function of angiotensin converting enzyme, chymase, and angiotensin II in the human radial artery and internal thoracic artery. Ann Thorac Surg 2000;70:2054–63.
[13] Chardigny C, Jebara VA, Acar C, Descombes JJ, Verbeuren TJ, Carpentier A, Fabiani JN. Vasoreactivity of the radial artery – comparison with the internal mammary and gastroepiploic arteries with implications for coronary-artery surgery. Circulation 1993;88:115–27.
[14] He GW, Yang CQ. Radial artery has higher receptor-mediated contractility but similar endothelial function compared with mammary artery. Ann Thorac Surg 1997;63:1346–52.
[15] Wei W, Yang CQ, Furnary A, He GW. Greater vasopressin-induced vasoconstriction and inferior effects of nitrovasodilators and milrinone in the radial artery than in the internal thoracic artery. J Thorac Cardiovasc Surg 2005;129:33–40.
[16] Dunser MW, Mayr AJ, Ulmer H, Knotzer H, Sumann G, Pajk W, Friesenecker B, Hasibeder WR. Arginine vasopressin in advanced vasodilatory shock – a prospective, randomized, controlled study. Circulation 2003;107:2313–9.
[17] Budzyn K, Marley PD, Sobey CG. Targeting Rho and Rho-kinase in the treatment of cardiovascular disease. Trend Pharmacol Sci 2006;27:97–104.
[18] Wickenden AD, Grimwood S, Grant TL, Todd MH. Comparison of the effects of the K+-channel openers cromakalim and minoxidil sulfate on vascular smooth muscle. Br J Pharmacol 1991;103:1148–52.
[19] Chester AH, Marchbank AJ, Borland JA, Yacoub MH, Taggart DP. Comparison of the morphologic and vascular reactivity of the proximal and distal radial artery. Ann Thorac Surg 1998;66:1972–6.
[20] Apostolidou IA, Skubas NJ, Despotis GJ, Kallinteri E, Hogue CW, Lappas DG, Barner HB. Occurrence of myocardial ischemia immediately after coronary revascularization using radial arterial conduits. J Cardiothorac Vasc Anesthes 2001;15:433–8.
[21] van Son JAM, Smedts F, Vincent JG, Vanlier HJJ, Kubat K. Comparative anatomic studies of various arterial conduits for myocardial revascularization. J Thorac Cardiovasc Surg 1990;99:703–7.
[22] Sadaba JR, Mathew K, Munsch CM, Beech DJ. Vasorelaxant properties of nicorandil on human radial artery. Eur J Cardiothorac Surg 2000;17:319–24.
[23] Stojnic N, Gojkovic-Bukarica L, Peric M, Grbovic L, Lesic A, Bumbarisevic M, Heinle H. Potassium channel opener pinacidil induces relaxation of the isolated human radial artery. J Pharmacol Sci 2007;104:122–9.
[24] Hizume T, Morikawa K, Takaki A, Abe K, Sunagawa K, Amano M, Kaibuchi K, Kubo C, Shimokawa H. Sustained elevation of serum cortisol level causes sensitization of coronary vasoconstricting responses in pigs in vivo – a possible link between stress and coronary vasospasm. Circ Res 2006;99:767–75.
[25] Fulton D, Gratton JP, Sessa WC. Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? J Pharmacol Exp Ther 2001;299:818–24.